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14. ABSTRACT
Receptor Tyrosine Kinase (RTK) activation triggers PI3 Kinase dependent Akt survival signaling. At the onset of this project, no genes involved in the feedback regulation of the PI3K – Akt signaling branch were known. In order to identify genes involved in the intrinsic negative feedback regulation of the activity of PI3K-Akt in *vivo*, I used *Drosophila* as a model system and performed unbiased cell based genome wide screens employing double stranded RNA interference (dsRNAi), measuring Akt phosphorylation as a readout for pathway activity. The results indicate that the activity of PI3K-Akt is indeed subject to feedback regulation, which is exerted by the Tsc1/Tsc2-Rheb-Tor pathway in a S6K dependent manner. This feedback control is not only present in tissue culture cells, but is also deployed during *Drosophila*development and is conserved in mammals. In addition, the data suggests a second regulatory connection between the PI3K and Tor signaling pathways via a dynamic equilibrium between the two distinct Tor containing complexes. Therefore, inhibition of Tor inevitably causes elevated levels of Akt activity. The results warrant caution to rely on the Tor inhibitor rapamycin and its derivatives as a single agent in anti cancer therapy.

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Cell signaling, Receptor Tyrosine Kinase Inhibitor, Transcriptional Feedback Inhibition, Molecular Diagnostic Marker, Therapeutic Agent

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Introduction

HER2/Neu signal transduction and breast cancer

In the course of neoplastic transformation, normal cells acquire the ability to escape the homeostatic mechanisms controlling growth and proliferation present in metazoans. In humans, this occurs as a multistep process, where evasion of apoptosis and acquisition of constitutive growth factor signaling, among others, culminate in the malignant cancer phenotype [1].

The HER2/Neu Receptor Tyrosine Kinase (RTK) is overexpressed in 20-30% of breast tumors and is found even more frequently associated with Ductal Carcinoma *in situ* [2]. Most importantly, high HER2/Neu correlates with a low disease-free as well as overall survival, highlighting the importance of understanding the underlying molecular mechanisms of HER2/Neu mediated transformation for the improvement of current therapies.

The PI3K-Akt signal transduction pathway has been described to have a pivotal role downstream of RTKs. In response to growth factors, RTK activation results in PI3K dependent activation of Akt [3]. HER2/Neu potently activates PI3K-Akt dependent signal transduction pathways [4-6]. The recent discovery of frequent oncogenic mutations in the PI3K gene in breast cancer patients further highlights the key position taken by the PI3K-Akt signaling pathway in malignant disease [7-15]. Furthermore, upregulation of Akt is a predictor of a poor disease outcome in least in some disease contexts [16,17].

Conservation of PI3K-Akt signal transduction in Drosophila

The PI3K-Akt signal transduction pathway is highly conserved between man and fly. It consists of the orthologs of various RTKs like EGFR, the Insulin Receptor (InR), or PVR, the PDGF Receptor ortholog, the adaptors of the Drk, Shc and IRS families (Chico), Phosphotidylinositol 3 kinase (Dp110) and its adaptor p60, the PIP3 phosphatase Pten, the constituents of the *Drosophila* TORC2 complex Tor and Rictor [18], PDK1 and Akt [19] (Figure 1). There is no known genetic redundancy within the PI3K-Akt signaling pathway. Each molecular function is encoded by a single gene. This makes *Drosophila* a formidable model organism to study highly conserved signaling processes.

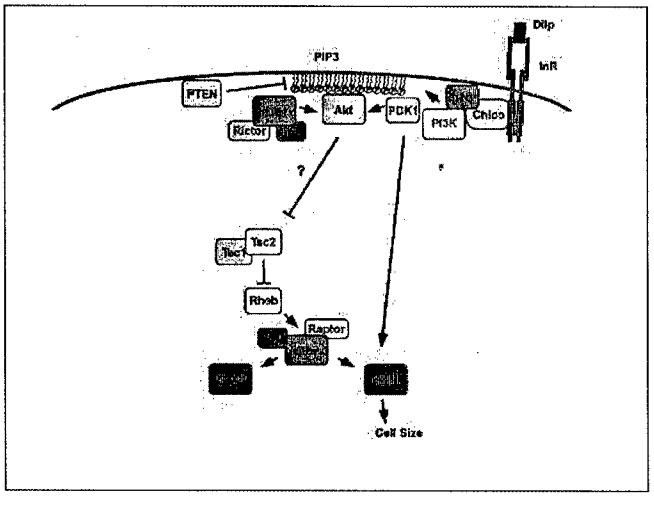

Figure 1: The *Drosophila* PI3K-Akt signaling pathway.
Drosophila Insulin-like peptides bind to and stimulate the activity if the InR receptor tyrosine kinase. The signal is transduced by the adaptor protein Chico and PI3Kinase, generating the phosphatidylinositol PIP3. The PH domain containing kinase PDK1 and the components of TORC2 (Tor, GβL, Rictor) activate Akt by phosphorylation. The dTsc1/dTsc2 complex negatively controls TORC1 and dS6K by its GAP activity towards the small GTPase Rheb. dS6K requires at least two independent phosphorylations to become active, one by TORC1 (Tor, GβL, Raptor) and one by PDK1. dS6K exerts negative feedback regulation to the upstream part of the pathway. In *Drosophila*, the link between Akt and dTsc2 is controversial[42].

Not surprisingly, genetic studies in *Drosophila* have highlighted the evolutionary conserved role that this pathway plays in controlling cell proliferation as well as cell size. Manipulation of the PI3K-Akt signaling activity *in vivo* results in altered sizes of the whole fly and of its organs in a cell autonomous manner [20]. These observations are in complete agreement with observations made in mammalian tissue culture as well in mice bearing mutations in either the Insulin Receptor [21], PI3K alpha and PTEN [22,23] or Akt3 [24], where investigators

reported a pronounced reduction of cell size and numbers in multiple tissues when Insulin signaling is impaired, while cell proliferation as well as cell size increases when the pathway is derepressed.

More recently, a second signal transduction pathway has emerged from a series of genetic studies in *Drosophila* connecting individual gene products to control cell growth, some of them long known. The pathway consists of the orthologs of the Tsc1 and Tsc2 tumor suppressor complex, the small GTPase Rheb, the components of the TORC1 complex Tor (Target of Rapamycin), GβL/Lst8 and Raptor, and *Drosophila* S6K. As in mammals, the Tsc1/Tsc2 complex directly regulates the activity of Rheb via the GTPase activating protein (GAP) domain of Tsc2 [25-27]. This link was originally discovered in *Drosophila* and subsequently confirmed in mammals [28-31]. *Drosophila* Rheb controls the activity of the Tor - Raptor complex (TORC1), probably by a similar mechanism as in mammals, by directly binding to Raptor [32,33]. In mammals as well as *Drosophila*, Tor is strictly required for the activation of S6K by means of direct phosphorylation of the S6K linker region. Similar to the Insulin signaling pathway, the Tsc1/Tsc2 - Rheb - Tor - S6K signal transduction pathway is highly conserved across phyla in terms of its constituents, the associated biochemical activities, their hierarchical organization and the associated physiological roles. Both, in mammals as well as in flies, the Tsc1/Tsc2 - Rheb - Tor - S6K controls cell growth and proliferation [34-36].

Initially, the PI3K-Akt and the Tsc1/Tsc2 - Rheb - Tor - S6K signaling cassettes were viewed as parallel pathways. However, in *Drosophila* as well as in mammals, they share at least three molecular connections. (1) PDK1 is required for the activation of S6K as well as Akt [37-39]. (2) Tsc2 is subject to direct inhibitory phosphorylation by Akt in tissue culture experiments [40-42]. (3) Tor is part of both complexes phosphorylating S6K (TORC1) and Akt (TORC2) [18]. In light of the genetic and biochemical studies in mammals and *Drosophila*, the previously held view that the Insulin and Tsc1/Tsc2 - Rheb - Tor - S6K signaling constitute two independent pathways has been revised to portraying them as two branches of a highly connected, interdependent signal transduction network.

Aim of this study

Studies performed in *Drosophila* have revealed a tight temporal and spatial control of signal transduction circuits activity by negative feedback circuits [43,44]. Based on the significant molecular conservation of the signal transduction machinery between man and the fly, it was hypothesized that negative feedback circuits regulating c-ErbB2 signaling pathways are present in humans. Additionally, this intrinsic feedback control might be inactivated by mutation in breast cancer patients, thereby contributing to the poor clinical outcome of c-ErbB2 positive breast cancers.

The purpose of this work is the identification and characterization of proteins involved in intrinsic negative feedback loops autoregulating Receptor Tyrosine Kinase activity in *vivo*. At the onset of this study, no genes involved in feedback regulation of the PI3 Kinase - Akt/PKB signaling branch have been described yet, although the presence of feedback regulation has been evident in *Drosophila* [45,46].

Approach

In *Drosophila*, approximately 75% of the ~14.000 predicted genes lack a functional annotation [47]. Given the availability of complete genome sequences and the development of technologies for targeted disruption of transcripts through double stranded RNA mediated interference (dsRNAi) [48], it is now possible to conduct unbiased genome-wide screens for gene function by screening a dsRNAi library covering every open reading frame of the entire *Drosophila* genome [49]. Therefore, to identify direct and feedback-regulators of Akt activity on a genome-wide scale, I set out to perform cell based genome wide screens employing dsRNAi.

The activation of Akt requires its dual phosphorylation: One on the kinase domain of Akt (mediated by PDK1) [50] and the second on the C-terminal hydrophobic motif of Akt by the TORC2 complex [18]. Both phosphorylation events are essential for the kinase activity of Akt towards its substrates, and phosphorylation of the hydrophobic motif (Ser 505 in *Drosophla*) closely reflects the activity of Akt [51]. To gain insight into novel

mechanism of Akt regulation I combined the unbiased power of genome-wide dsRNAi screening with reading the Akt activity state by its phosphorylation status, using a phospho-specific antibody.

Results

In collaboration with Cell Signaling Technologies (Beverley, MA), I generated a phospho-specific Akt antibody against *Drosophila* Akt and thoroughly tested its specificity. I developed a high-throughput, cell based assay to screen for dsRNA either enhancing or suppressing endogenous Akt phosphorylation in an unbiased fashion and performed growth factor stimulated and non-stimulated genome wide dsRNAi screens. I have build a robust data acquisition and normalization method which can be uniformly applied to any screen using phospho-specific antibodies as readouts for pathway activity. One central result emerging from the screens is the feedback regulation of Akt by the Tsc1/Tsc2-Tor-S6K signaling branch. This regulatory mechanism is not only present in *Drosophila* tissue culture cells used for the RNAi screens, but also in the developing *Drosophila* organism to restrain Akt activation over a certain threshold. Similar findings in mammalian tissue culture and mouse models confirm the conservation of this mechanism [52-56] and point to the necessity to supplement current clinical trials of the anti-tumor activity of mTor inhibitor rapamycin and its derivatives with inhibitors of Akt [57].

Body

A readout for Drosophila Akt phosphorylation and activity

We aimed to establish an assay for regulators of insulin signaling in *Drosophila,* which would be adaptable to perform a cell-based genome-wide RNAi screen. To identify suitable tools, we tested 64 commercially available phospho-antibodies generated against conserved mammalian proteins of the insulin signal transduction pathway by western blot and immunofluorescence on *Drosophila* Kc_{167} cells (data not shown). While approximately 10% of the antibodies tested cross-reacted on Western blots in an insulin-dependent manner, none of them recognized an insulin induced antigen on *Drosophila* Kc_{167} cells. To create a robust antibody-based readout tool for insulin signaling, we generated a phosphospecific antiserum recognizing the phosphorylation of the C-terminal hydrophobic motive of *Drosophila* Akt, Serine 505 or Ser 586 of the short and long splicoform, respectively (hereafter referred to as anti P-Akt).

Phosphorylation of the hydrophobic motif closely reflects activity of Akt [51]. Stimulation of Kc_{167} cells with insulin for 10 minutes induced a robust P-Akt signal, which was strongly suppressed when known components of the insulin signaling cascade like InR, Chico, the catalytic subunit of PI3-Kinase PI3K92E and Akt itself were silenced by RNAi (Figure 2 A-E').

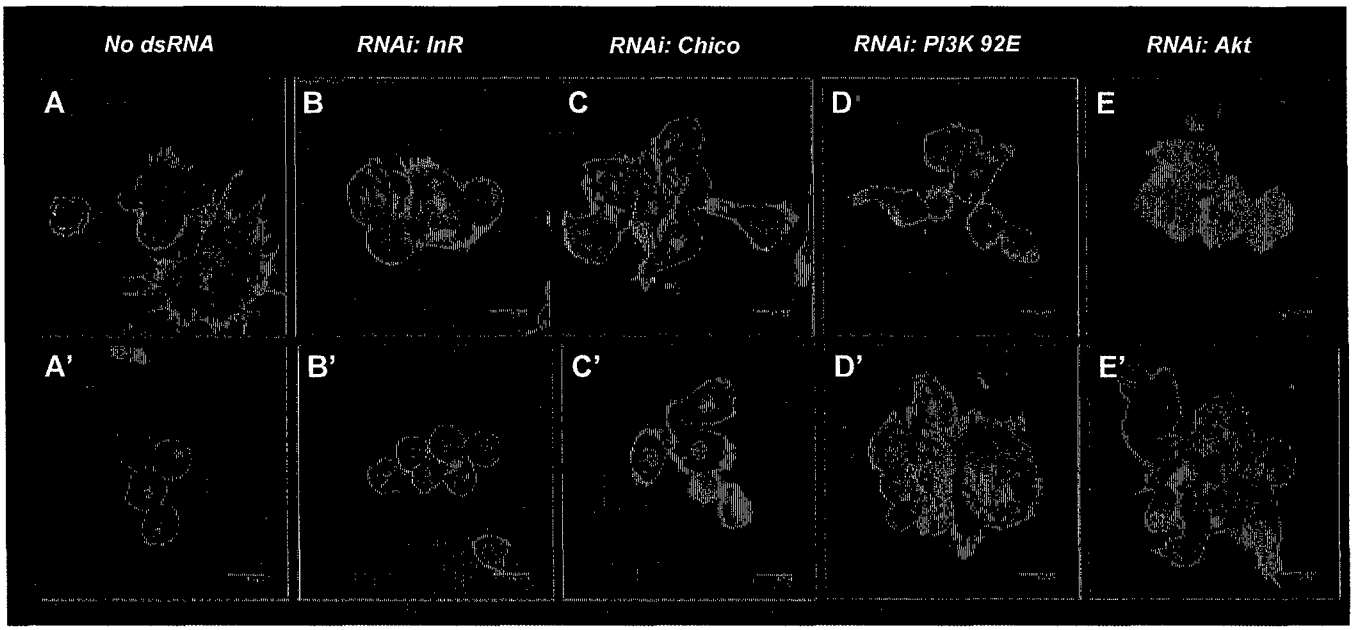

Figure 2. Specificity of the anti P-Akt (Ser505) antibody. (A-E') *Drosophila* Kc_{167} cells stained with DAPI (blue) anti P-Akt (Ser505) antibody (green) and anti α-tububin (red) at baseline (A'-E') or 10 minutes of Insulin stimulation (A-E). Cells were RNAi treated as described in experimental procedures using no dsRNA (A & A'), InR dsRNA (B & B'), Chico dsRNA (the IRS ortholog, C & C'), PI3K93E dsRNA (the caltalytic subunit of the class I_A PI3-Kinase, D & D') and Akt (E & E'). Please note that large polynucleated cells are resistant to the Insulin stimulus (A)

We next asked whether the anti P-Akt antibody detected differences in Akt phosphorylation in the 3rd instar imaginal disc induced by expression of dominant negative insulin receptor (InR^{DN}) or a constitutively active catalytic subunit of PI3K ($PI3K^{CAAX}$) using the UAS-Gal4 expression system [58]. Staining of *wt* imaginal wing discs revealed no pattern of P-Akt associated with compartments or compartment boundaries (not shown). We used ap-Gal4 to drive expression of Inr^{DN} and $PI3K^{CAAX}$ concomitant with membrane tagged GFP in the dorsal compartment and compared levels of P-Akt to ventral cells as controls (attached manuscript Figure 1F-G'). As expected, expression on InR^{DN} resulted in a reduction of P-Akt (attached manuscript Figure 1F and F') and $PI3K^{CAAX}$ expression drastically increased the levels of P-Akt when compared to ventral control cells (attached manuscript Figure 1G and G'). Western blotting experiments of extracts from Kc_{167} cells pretreated

with dsRNAs against InR, PI3K92E, and Akt confirmed the specificity found in the immunohistochemical stainings on cells and *Drosophila* tissue (attached manuscript Figure 1H). Surprisingly, RNAi against PDK1, the Akt Ser308 kinase ortholog in Drosophila, enhanced P-Akt Ser 505 phosphorylation rather than suppressing it. However, this finding is consistent with mammalian tissue culture results [59,60]. Taken together, our data show that anti-PAkt faithfully detects Akt phosphorylation in cell culture and in vivo.

A Pilot dsRNAi screen for regulators of Akt

In order to use the Antibody as a screening tool for libraries of double stranded RNA interference (dsRNAi) treated cells, a high throughput protocol using 384-well microtiter plates for culturing, dsRNA treating, P-dAktSer505 antibody staining and microscopic analysis has been established. Treatment of cultured *Drosophila* cells with gene-specific dsRNA has been shown to exclusively deplete the mRNA of the targeted gene, concomitant with a sharp decrease of the related protein level [48].

Initially, 94 genes encoding for Kinases, Phosphatases, small GTPases as well as other signaling molecules have been selected and tested as a pilot screen. Image acquisition was facilitated by a Nicon Autoscope, a microscope with a motorized stage and Metamorph automated image acquisition software. Image analysis was performed "by eye".

While successful to a certain extend, the pilot screen showed the absolute necessity for a faster and more quantitative assay if full genomes with 21.500 dsRNAs were to be screened in replicates, especially when the screens are done with various different stimuli.

A fast and quantitative cell based high throughput assay: The Cytoblot

The cytoblot / In Cell Western is a chimera combining parts from immunohistochemical staining of tissue culture cells and simple western blot techniques into a high throughput format [61].

The cells are seeded in 384 well plates and grown in the presence of various dsRNAs to confluency for a three day period. Then, the cells are fixed and immunohistochemically stained with anti P-Akt antiserum. However, instead of a fluorophor-conjugated secondary antibody, Horseradish Peroxidase conjugated secondary Antiserum is used to detect and quantify the primary anti P-Akt antibody, very much alike standard Western Blotting. Detection of P-Akt is facilitated in a 384 well plate reader quantifying the chemiluminescence emitted after addition of Western Blot substrate.

The individual dsRNAs can influence the luminescent signal in two ways: Either the dsRNA targets and represses the expression of a gene required for the phosphorylation or dephosphorylation of Akt. Alternatively, the dsRNA is eliciting an effect on the cell cycle / cell death / cell adhesion machinery, leading to an altered cell number and consequently an altered chemiluminescent signal from this well [49]. The former dsRNAs are the "specific hits" the screen is designed for, the latter would represent a distinct form of "noise". In order to avoid false positive hits caused by cell number variation, the luminescence from each well is normalized to a cell count value of the very same well. This is facilitated by fluorescently staining nuclear DNA and the subsequent recording of the fluorescent value in a 384 well plate reader. Cell number titration experiments showed that the fluorescent values are proportional to the number of cells in each well.

The cytoblot is a fast and robust assay. While automated microscopy of a single plate takes 2.5 – 3 hrs, a dual Luminescence and Fluorescence read takes 6 minutes. Furthermore, microscopic analysis acquires data from a fraction of a single 384 well surface, while the plate reader based cytoblot reads >90% of the well.

All these advantages of the HRP-coupled version of the cytoblot notwithstanding, the first two genome wide screens which were performed at baseline (not stimulated with growth factor) unfortunately showed a high noise level and strong plate-to-plate variability of the average and median luminescence levels. This made the following data analysis cumbersome and called for an improved version of the assay.

In the cytoblot version currently used, the anti P-Akt antibody is detected and quantified by an AlexaFluor 680 coupled secondary antibody using an Aerius near-infrared plate reader. In this setting, the dynamic range of P-Akt variation is more than 10 fold (HRP coupled detection: 5-7 fold) and the variance less

than 4% (HRP coupled detection: 20%) of the measured values. Moreover, the assay plate does not suffer from the decaying activity of an enzyme over time and therefore can be read multiple times up to two months after the assay was performed without loss in data quality.

Genome-wide RNAi screen for regulators of P-Akt

In order to search for components of the insulin signal transduction pathway and to identify novel regulatory inputs, we chose Cytoblotting /In Cell Western (Figure 3A) as a fast and quantitative cell-based high throughput assay. Cells were grown in 384 well plates and, after three days in the presence of various dsRNAs, were fixed and immunostained with anti-P-Akt antiserum. Bound primary antibody was quantified by near Infra-Red dye conjugated secondary antibody in a 384 well plate reader.

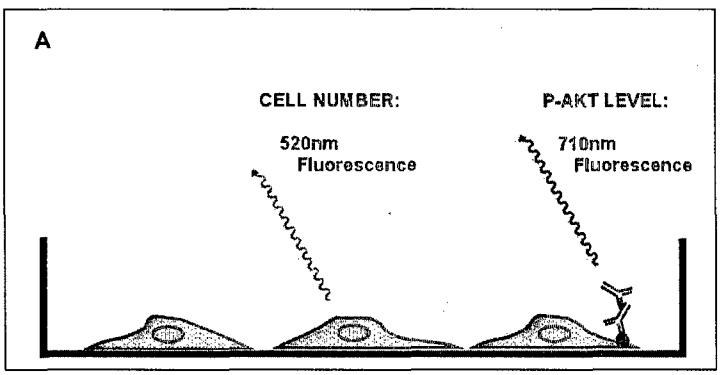

Figure 3: The Cytoblot. Cells are seeded in a 384 well plate and grown for 3 days in the presence of various dsRNAs. A single well of a 384 well plate is depicted here. After serum starvation, cells are either stimulated with insulin for 10 minutes or left untreated for baseline assessment. Cells are fixed and stained with a combination of anti P-Akt, AlexaFluor 680 Dye (shown in red) coupled secondary antibody and a DNA dye (show in green). Data are acquired in 384 well plate readers from LiCor (Aerius, 710 nm) and Molecular Devices (Analyst GT, 520nm).

Values were normalized to total cell counts as measured by fluorescent nuclear staining as follows: A robust linear regression was performed on the log2(P-Akt/NucFl) values of each individual screen plate, and residuals from the log2(P-Akt/NucFl) values to the regression line were calculated. All residuals of each genome wide screen were pooled and a cell number dependent error model was developed and used to determine locally weighted standard deviations (SD) and averages in dependence of cell number. Z-scores using these two parameters were calculated, expressing the deviation from the local average value in SDs. All Z-scores were corrected against position effects by setting the Mean Z-Score of each individual well position across one genome wide screen replicate to zero.

The dependence of the false positive rate on a threshold Z-score (cut off) was analyzed using two external 384 well plates containing cell number gradients which were processed in parallel with the 58 screening plates as well as 416 wells in the screening plates which did not contain any dsRNA. At the cut off value chosen for my screens (+/- 2SDs), the false positive rate is approximately 2.5%.

Genome-wide RNAi screens for modulators of Akt phosphorylation were performed in replicates without stimulation (to determine baseline levels) and after 10 min. of insulin stimulation. Employing a cutoff value of +/- 2 Standard Deviations (SDs) relative to the screen average, we found that 126 dsRNAs resulted in the suppression of Akt phosphorylation while 172 enhanced it. Importantly, we identified 9 out of the 11 known components upstream of Akt, validating the reliability of this method (attached manuscript Figure 2B and C). PDK1 RNAi resulted in an upregulation of P-Akt Ser505, consistent with the results shown in the attached manuscript figure 1H. The two known regulators not to be found in the screens were Chico and Pten, which did not reach above the cutoff threshold.

Surprisingly, we found that Akt phosphorylation is sensitive to the interference of the Tsc1/Tsc2-Tor-S6K signaling branch (attached manuscript Figure 2B and 2C), classically viewed as downstream components of Akt [35,36,62]. At first glance this seems counter-intuitive as phosphorylation of Akt should reflect events regulated by upstream elements of the signal transduction pathway but not by downstream regulators. However, we found that dsRNAs against downstream mediators required positively for insulin signal transduction like the small GTPase *Rheb*, the TORC1 component *Raptor* and *S6K* induced ectopic phosphorylation (activation) of Akt in the absence of an insulin stimulus. Conversely, dsRNAs against the negative regulators *Tsc1* and *Tsc2*

suppressed the P-Akt signal when the pathway was in its active (insulin stimulated) state. These results can be interpreted in a model of an inhibitory feedback signal mediated by the components downstream of Akt, namely Rheb, Rictor, Tsc1/2 and S6K. When the negative feedback is disrupted, ectopic activation of Akt is triggered.

Feedback regulation of Akt phosphorylation by the Tsc1/Tsc2-Tor-S6K signaling branch

To confirm the results of the genome-wide RNAi screens, we re-synthesized independent dsRNAs to most of the known pathway members and retested their effects on P-Akt in seven replicates by cytoblot using an external standard for normalization against cell number dependent effects (attached manuscript Figure 3A and B). An external standard consisting of 768 values of non-RNAi treated cells covering the whole spectrum of cell densities was used to determine cell number dependent averages and SDs to calculate experimental Z-Scores of RNAi treated wells. All P-Akt values of non-stimulated cells are normalized using a baseline standard curve (the average non-treated, non-stimulated experiment scores zero). For the insulin stimulated case, the P-Akt values of insulin treated cells are normalized using a standard curve derived from insulin stimulated cells (the average non-treated, insulin stimulated experiment scores zero).

dsRNAs against *GFP*, *MEKK1/4*, *Thread* and *CSK* were used as negative controls, *Pten* dsRNA as positive control. As found in the genome-wide screens, removal of the negative regulators Tsc1 and Tsc2 resulted in a suppression of P-Akt when insulin is present, while knock down of S6K elevated P-Akt at baseline conditions. This demonstrated that disrupting the feedback loop is most effective under a non-stimulated condition.

In order to test the feedback hypothesis by different means than RNAi, we inhibited the activator of S6K, TOR, by two independent strategies (attached manuscript Figure 3C): First, in a chemical approach, we exposed cultured cells to the small molecule Rapamycin, an effective inhibitor of TOR in *Drosophila* [63,64] and second, in a metabolic approach, we starved cultured cells in amino acid free media, thereby potently inhibiting TOR activity [45,46]. Amino acid starvation, as well as rapamycin-induced TOR inhibition, leads to a highly significant increase in P-Akt compared to control cells treated with amino acid containing medium or methanol solvent control, respectively. This confirmed the RNAi data obtained from the genome-wide screen and validates the idea of a negative feedback loop auto-regulating the activity of the pathway after stimulation.

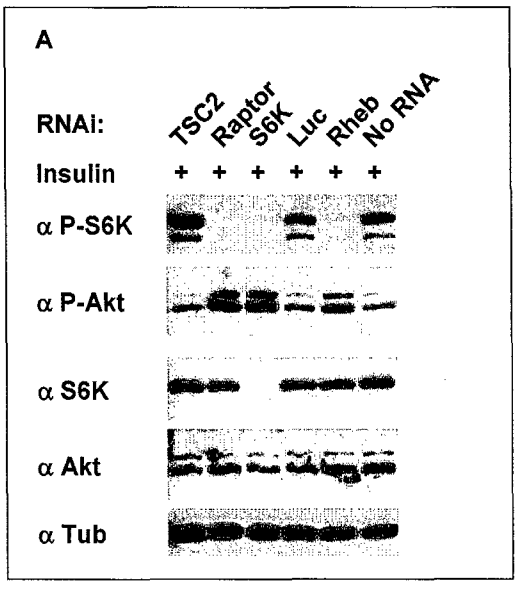

Figure 4. Inhibition of dS6K results in derepression of Akt by inhibition of InR. (A) Immunoblotting of crude lysates prepared from *Drosophila* Kc_{167} cells after 10 min of Insulin stimulation treated with dsRNAs as indicated. Non-RNAi treated and luciferase (luc) dsRNA treated cells were used as negative controls.

We next asked if the inhibitory potential of S6K correlates with its activity, using the phosphorylation of *Drosophila* S6K linker region Serine 398 (homologous to mammalian S6K1 Ser389) as readout (Figure 4A). Total lysates of *Drosophila* Kc167 cells pretreated with dsRNAs against *Tsc2*, *Raptor*, *S6K* and *Rheb* were analyzed for S6K and Akt phosphorylation. Cells treated with dsRNAs against luciferase and non-RNAi treated cells served as negative controls (Figure 4A, lanes 4 and 6). Enhancement of P-Akt strongly correlated with suppression of the phosphorylation status of S6K, with a clear elevation of Akt phosphorylation when Rheb, Rictor or S6K expression was knocked down by RNAi.

In order to address how S6K mediates its feedback inhibition to Akt, we triggered ectopic Akt phosphorylation by RNAi against S6K and asked whether other components of the insulin signaling pathway were required for this effect by removing them in addition to S6K (attached manuscript Figure 4B). Removal of S6K resulted in a robust enhancement of P-Akt of 4 SDs above non-RNAi treated control cells. Additional

RNAi against control genes like *GFP*, *CSK* or *MEKK1/4* did not significantly change the P-Akt value, confirming that combined RNAi against two genes does not influence the efficiency of the RNAi silencing. As positive controls, co-RNAi of S6K and Pten resulted in an elevation of P-Akt levels, while dsRNA treatment against S6K and Akt reduced P-Akt to levels of unstimulated, non-RNAi treated cells. Interestingly, RNAi against the positive regulators InR and PI3K, did suppress the S6K RNAi induced ectopic Akt phosphorylation, indicating that the S6K dependent feedback inhibition is mediated by these two components. However, co-RNAi of Chico with S6K did not reduce P-Akt. This is in contrast to recent reports in mammals showing S6K dependent regulation of IRS transcription, phosphorylation and protein stability [54-56]. Our results suggest that the feedback regulation is routed through InR and PI3K in a Chico independent manner.

A S6K independent TORC1 and TORC2 interconnection

One of the most striking results emanating from the analysis of the non-stimulated genome-wide P-Akt RNAi screen is that *Raptor* dsRNAi scores a more than two-fold higher P-Akt value than S6K (attached manuscript Figure 2B). As the negative feedback loop is mediated through S6K, and dsRNA against S6K is very efficient (Figure 4A, lane 3 and attached manuscript Figure 5E, lane 7), we could not explain this effect by an exclusive involvement of Raptor in S6K dependent P-Akt regulation. To verify the results and exclude that the higher Z-Score of Raptor RNAi is based on a effect rooted in the presence of these two dsRNAs on different screening plates, we performed anti P-Akt immunohistochemical staining on Kc_{167} cells treated with dsRNAs against luciferase (negative control), Pten (positive control), S6K and Raptor (Figure 6 A-D). Confocal imaging and digital processing using identical settings confirmed Raptor RNAi triggering the highest levels of ectopic Akt phosphorylation when compared to S6K, Pten and luciferease. This result prompted the hypothesis that Raptor participates in an additional regulatory loop interconnecting the InR-PI3K-Akt and Tsc1/Tsc2-Tor-S6K branches.

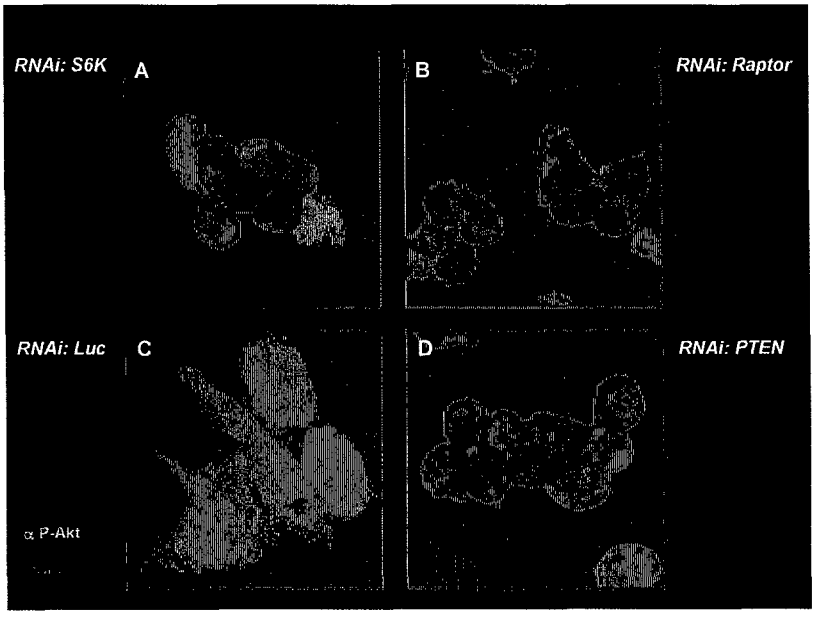

Figure 5. Reciprocal regulation of dS6K phosphorylation by TORC1 and TORC2. (A) Single confocal section of dS6K, (B) Raptor, (C) Luciferase and (D) Pten RNAi treated *Drosophila* Kc_{167} cells stained with DAPI (blue) P-Akt (Ser505) antibody (green) after 10 minutes of Insulin stimulation. Images were recorded and processed using identical conditions. Note the highest level of P-Akt (Ser505) in the Rictor dsRNA treated cells.

We therefore tested the phosphorylation status of Akt and S6K in the presence or absence of functional TORC1 and TORC2 (attached manuscript Figure 5E). Both complexes are constituted by Tor and GbL as common components, and by Raptor (TORC1, responsible for S6K 398 phosphorylation [45,65,66] and Rictor as well as Avo1 (TORC2, the Akt Ser505 kinase [18] as unique and defining factors. As shown in Figure 5 of the attached manuscript, the phosphorylation of Akt is elevated when the expression of the specific component of TORC1, Raptor, is silenced by RNAi, while P-Akt is strongly suppressed when the unique TORC2 molecules Rictor and Avo1 are RNAied. Conversely, the phosphorylation of S6K is strongly reduced when cells are treated with *Raptor* and *Tor* dsRNAs. Most interestingly, phospho-S6K levels are elevated when TORC2 function is disrupted by RNAi against Avo1 and Rictor, however to the same extent as seen in the case of P-Akt and Raptor RNAi. We

interpret the result in favor of a regulatory connection between TORC1 and TORC2, where disruption of one complex elevates the activity levels of the other.

Feedback regulation of P-Akt in the Drosophila wing imaginal disc

While our data clearly indicated regulation of P-Akt in Drosophila Kc_{167} cells by a feedback loop, we wanted to test whether this mechanism is also conserved in the developing fly. Previous experiments have shown that connections within the insulin signaling pathway observed in *Drosophila* tissue culture cells are either not physiological relevant or are absent in the whole organism [38,40,42]. In order to test if P-Akt is regulated by the Tsc1/Tsc2-Tor-S6K signaling branch *in vivo*, we used the UAS-Gal4 system [58] to co-express Tsc1 and Tsc2 in the dorsal compartment of the third instar imaginal wing disc under the control of ap-Gal4 (Figure 6).

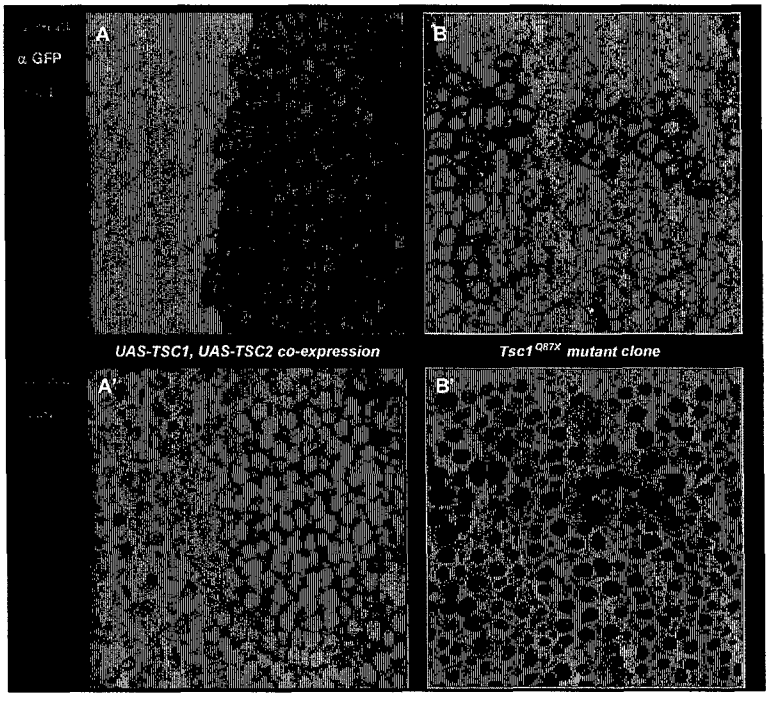

Figure 6. Feedback regulation of Akt in the developing wing imaginal disc of *Drosophila*. (A-B') Single tangential optical sections of 3rd instar wing imaginal discs. Wing imaginal discs are stained with DAPI (blue), P-Akt (Ser505) antibody (red) and anti-GFP (green). (A, A') A magnified view on the dorso-ventral boundary at the wing pouch is shown. GFP expression (green) marks the expression domain of *apterous-Gal4* driver and the *UAS-Tsc1, UAS-Tsc2* expression constructs. (B, B') *Tsc1Q87X* homozygous mutant clones located at the wing primordium. Mutant clones are marked by the expression of GFP (green). A' and B' show DAPI and P-Akt channels only, the compartment boundary (A') or the line tracing of the clones (B') is marked with by a white line.

The co-expression of Tsc1 and Tsc2 has been shown to strongly inhibit TORC1 and S6K activity, leading to reduced cell and organ size [67-69]. Therefore, if the feedback loop was intact in vivo, we expected an upregulation of P-Akt when compared to ventral control cells. Indeed, we detected a clear increase of P-Akt in Tsc1, Tsc2 overexpressing cells (Figure 6A'). To ask the converse question if P-Akt levels were reduced when Tsc1/Tsc2 function is reduced, we induced homozygous mutant clones of $tsc1^{Q87X}$, a null allele, in the wing disc and analyzed Akt phosphorylation by staining with anti P-Akt. Mutational disruption of either Tsc1 or Tsc2 results in loss of function of the Tsc1/Tsc2 tumor suppressor complex and should therefore result in the converse P-Akt phenotype than the overexpression experiment. We found reduced P-Akt levels in $tsc1^{Q87X}$ mutant tissue when compared to *wt* control tissue (Figure 6B'). In conjunction with the Tsc1/Tsc2 overexpression data this confirmed that P-Akt is regulated by feedback signaling *in vivo*.

We next addressed the question whether Tor was required for Akt Ser 505 phsophorylation in vivo. Our results by Western blotting in tissue culture experiments showed only a mild effect of Tor RNAi on Akt phosphorylation, possibly by the antagonizing effects of removing feedback inhibition concomitant with TORC2 disruption in a background of partial RNAi knockdown. Therefore, we induced $tor^{\Delta P}$ homozygous mutant clones in a *wt* as well as $Pten^{DJ189}$ mutant background [66]. It was noted before that loss of Tor function suppresses the loss of Pten [65,66]. We found that P-Akt is upregulated in $Pten^{Dj189}$ loss of function clones (attached manuscript Figure 7A and A'). This elevation of P-Akt was suppressed when *Tor* function is removed simultaneously with $Pten^{DJ189}$ (attached manuscript Figure 7B and B'). Clones homozygously mutant for the

$Tor^{\Delta P}$ allele showed a suppression of P-Akt in a *wt* background, however we observed this reduction only in clones that were induced early during larval life. In summary, we conclude that Akt phosphorylation is dependent on Tor function *in vivo* as its described role predicts [18] and that the regulatory feedback of Akt by the Tsc1/Tsc2-Tor-S6K signaling branch is conserved in *vivo*.

RNAi screening the insulin signaling circuitry

Initially, the genome-wide RNAi screens described here were designed to identify novel regulators acing upstream of Akt. However, the clear emergence of the whole Tsc1/Tsc2-Tor-S6K signal transduction branch (all known regulators scored above the cutoff threshold) as mediator of negative feedback regulation showed that the approach is versatile beyond the identification of the direct activators. However, the topology of the InR-PI3K-Akt and Tsc1/Tsc2-Tor-S6K signaling branches and the dual phosphorylation mechanism of Akt activation lead to some surprising results.

PDK1, although an activating kinase of Akt, receives scores marking it as a suppressor of P-Akt, a finding actually consistent with the literature [59,60]. This observation can be rationalized by two independent phenomena. First, Akt protein expression is upregulated in *PDK1* RNAi cells (attached manuscript Figure 1H). Second, PDK1 phosphorylation is targeted at the kinase domain, a site not recognized by the anti-P-Akt antibody directed against the hydrophobic motive used in this study. However, removal of PDK1 by RNAi gives rise to inactive Akt, which leads to inactive S6K (attached manuscript Figure 5E) and the disruption of the negative feedback inhibition to the upstream part of InR signaling. Therefore, the upregulation of hydrophobic motive phosphorylation results as a logic consequence of PDK1 expression silencing. Therefore, the classification of PDK1 as a suppressor is - although misleading - in accordance with the signaling circuitry displayed in Figure 7.

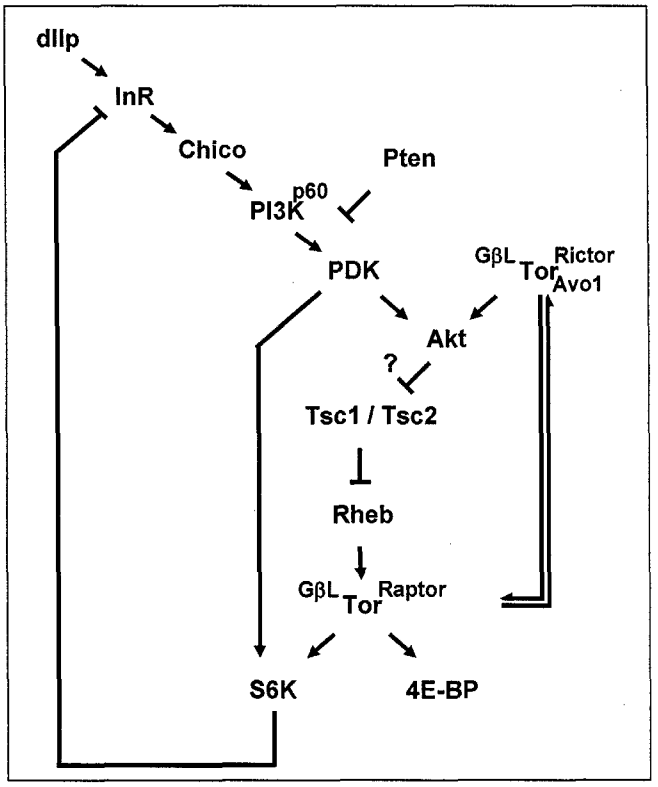

Figure 7. Model of the InR-PI3K-Akt and Tsc1/Tsc2-Tor-S6K signaling circuitry in *Drosophila*. dS6K elicits feedback inhibition via InR, resulting in Insulin resistance when highly active. *Drosophila* TORC1 and TORC2 complexes are connected by containing common components like Tor and GbL. In addition, disassembly of one TORC results in higher activity for the other, probably by a mass action equilibrium

From our screening effort, RNAi against Rictor and Avo1 lead to strong suppression of insulin induced phosphorylation of Akt, consistent with its described role as TORC2 component, the Akt hydrophobic motive kinase [18]. Components like Tor and GβL, constituents of both TORC1 and TORC2, come out of the screen as molecules required for Akt Ser505 phosphorylation, but with a clearly lower score. Why do the shared TORC components score at lower strength? RNAi against Tor and GβL have dual, antagonistic effects: In addition to reducing the TORC2 activity towards Akt, it weakens the TORC1 complex, silence S6K activity and therefore remove feedback inhibition. In flies, only Tor clones induced very early in larval development (translating to "old" clones at the third imaginal stage) show a reduction of endogenous P-Akt levels. A long protein half life of *Drosophila* Tor would account for this observation. In addition, only minimal levels of functional TORC2 complex might be sufficient for Akt Ser505 phosphorylation, presumably at the plasma membrane [18], while higher levels of TORC1 are required for S6K phosphorylation, making this complex more sensitive to RNAi induced disruption (attached manuscript Figure 5E).

Regulation of Akt phosphorylation in vivo

Our initial experiments on wing imaginal disc tissue expressing either activated PI3K or dominant negative InR displayed very high activation by $PI3K^{CAAX}$ expression, but a comparatively low suppression of P-Akt by InR^{DN} (attached manuscript Figure 1F-G'). We interpret this finding as a relatively low constitutive phosphorylation of Akt present in the 3rd instar imaginal wing disc, which has much more "headroom" for activation than for suppression. In agreement with this hypothesis, the relative upregulation of P-Akt overexpressing Tsc1 and Tsc2 is clearly higher than the suppression of P-Akt in $tsc1^{Q87X}$ homozygous mutant clones. However, we can not exclude that some unspecific background binding of the anti P-Akt antiserum contributes to the observed effect. In addition, our analysis provides only a snapshot into the developmental dynamics of Akt phosphorylation, and P-Akt levels might be higher at different times of development.

The elevated P-Akt levels of the Tsc1-Tsc2 co-expression experiment is a result clearly in favor of a conserved negative feedback regulation of Akt by the Tsc1/Tsc2 tumor suppressor complex *in vivo*. However, it is surprising at two levels. For once, Akt has been described as regulator of cell size, and akt^1 homozygous mutant clones show reduced cell size [70]. However, the outcome of Tsc1/Tsc2 co-expression is reduced cell size, despite the elevated levels of P-Akt [67-69]. The converse experiment highlights the same paradox: $tsc1^{Q87X}$ mutant cells have a larger cell size, despite lowered P-Akt levels (and activity). These results might indicate that for cell size, the only function of Akt is the regulation of Tsc1/Tsc2 activity. In support of this premise, no other Akt substrate (e.g. FOXO, Gsk3β) has been shown to elicit a cell size defect so far [71].

The second surprise lays in the fact that Tsc1/Tsc2 overexpression triggers ectopic Akt activation, which should however inactivate Tsc2 by phosphorylation and therefore lower the P-Akt phosphorylation status. If the Akt-Tsc2 link is of less physiological relevance as initially suggested [40,42], or if overexpressed Tsc1/Tsc2 localizes to a subcellular compartment where it escapes phosphorylation by active Akt but can elicit Rheb inhibition remains to be seen.

The TORC1-TORC2 connection

A centered view on the two Tor containing complexes TORC1 and TORC2 reveals the multiplicity of connections between the InR-PI3K-Akt and Tsc1/Tsc2-Tor-S6K branch. They are, at least in tissue culture systems and in some pathological states like cancer, molecularly closely connected by the direct phosphorylation of Tsc2 (a TORC1 regulator) by Akt (a TORC2 substrate) [18,41,52,53,68,72,73]. They share a second link comprised of S6K (a TORC2 substrate) negatively feeding back on the activity of the upstream part of the InR signaling pathway required for Akt activation [45,54-56]. Our data suggest the presence of a third link, where disruption of one TORC complex increases the activity of the other. In a strict sense, this can be interpreted as mutual inhibition of TORC1 and TORC2, but we propose an assembly/disassembly equilibrium between TORC1 and TORC2 as a mechanism, possibly driven by mass action law (Figure 7). Recent work in *S. cerevisiae* has shown that TORC2 disintegrates when cells are depleted from Avo1 or Avo3 (Rictor) [74]. If the requirements of Avo1 and Rictor for TOR complex 2 integrity are conserved higher eukaryotes, Tor/GβL would be free to be recruited into TORC1 complexes, giving rise to a higher level of TORC1 activity.

On initial inspection, the effect of Raptor removal on Akt phosphorylation seems to be much more drastic than the effect of Rictor RNAi on P-S6K levels (attached manuscript Figure 5E). However, the amplitude of P-Akt level change upon expression silencing of Raptor is bigger because Akt feedback inhibition is disrupted in addition to any putative shifts in the TORC1-TORC2 equilibrium. The relative differences of P-Akt levels from S6K to Raptor RNAi cells compared to changes of P-S6K in luciferase vs Rictor or Avo1 RNAi cells reveal a much more comparable effect. Nevertheless, the reciprocity of the TORC1-TORC2 equilibrium remains to be quantified.

The TORC2 complex has been suggested as attractive therapeutic target [57,75]. However, the tight interconnection of TORC1 and TORC2 warrants caution not inhibit one and activate the other with possible deleterious side effects.

Key Research Accomplishments

- A phospho-specific antibody against phosphorylated Ser505 of *Drosophila* Akt has been characterized and tested on western blotting and immunohistochemistry on endogenous Akt in tissue culture cells employing dsRNAi and chemical inhibitors.
- Protocol of assaying endogenous Akt phosphorylation in developing *Drosophila* organism in place. The phospho-specific Akt antibody is the tool of choice to analyse PI3K-Akt signaling *in vivo*.
- Quantitative high throughput protocol for screening dsRNAi treated tissue culture cells by Cytoblot / In Cell Western established. Method to normalize against cell number variation implemented.
- Establishment of a robust statistical strategy for scoring and ranking the effect of individual dsRNAs eliminating bias from screening plate position and non-linear effects of cell number variation.
- Completion of two replicates of genome wide dsRNAi screens for regulators and components of Akt phosphorylation under baseline, non-stimulated and under insulin stimulated conditions. Isolation of more than 200 genes which influence or regulate the Akt activity status, most of them with no known role in Akt signaling. The genetic and biochemical analysis of some of these genes is ongoing.
- I developed a secondary RNAi screening method using independently synthesized dsRNAs and employing an external standard to score the effect of individual dsRNAs on Akt phosphorylation.
- Description and analysis of regulatory feedback regulation within the PI3K-Akt and Tor signal transduction pathway using dsRNAi, chemical inhibitors (rapamycin) and metabolic perturbation (amino acid starvation) in a tissue culture model.
- Description and analysis of the regulatory feedback regulation within the Akt signal transduction pathway found in cell culture *in vivo* using defined genetic mutations in the PI3K and Tor signaling pathway. This shows the transferability of results from our cell culture model to the true *in vivo* setting and verifies the approach of RNAi screening as a global analysis tool.

Reportable outcomes

Please see attached manuscript in appendix. For the research community, the anti *Drosophila* P-Akt antibody is now available via Cell Signaling Technologies. I was invited to orally present this work at the plenary session of Keystone Symposium for Diabetes and Metabolic Disease in Keystone, Co in Jan 2005, the 46th annual Drosophila Research Conference in San Diego, Ca, the Drosophila RNAi Screening Center Symposium at Harvard Medical School in Boston, MA, the CHI conference on RNAi Pathway Analysis in Boston, MA and received my first personal invitations by Prof. George Thomas to speak at the Center for Genome Research in Cincinnati, Ohio and Millenium Pharmaceuticals in Cambridge, MA. I presented a poster at the Era of Hope Conference 2005 in Philadelphia. The work supported by this DoD Breast Cancer Fellowship enabled me to apply for and win a postdoctoral grant from the Tuberous Sclerosis Alliance which currently supports the continuation of this work.

Conclusions

Alterations of the activity of several components of the Akt signal transduction pathway are molecularly linked to proliferative diseases and especially breast cancer [64,76]. The Tor inhibitor Rapamycin and its derivatives are currently investigated in clinical trials as potential cancer drugs [75].

This study shows that inhibition of Tor signaling leads to elevated levels of *Drosophila* Akt activity by disrupting a negative feedback loop which emanates from Tor and inhibits Akt in a S6K dependent manner. The regulatory mechanism of Akt regulation by feedback control is conserved in mammalian tissue culture models [55,56]. Furthermore, disruption of Akt feedback control has been shown to worsen the outcomes of specific cancer types in mouse models of hepatic hemangiomas and several other tumor types [52,53].

It is therefore important to notice that disruption of Tor function by Rapamycin might not only disrupt the downstream events of signal transduction, but counterproductively might also ectopically activate Akt by suppressing feedback inhibition on Akt, with all its consequences to elevated cell survival [1]. Hence, it might be necessary to supplement current clinical trials of the anti-tumor activity of mTor inhibitor rapamycin and its derivatives with inhibitors of Akt to improve therapeutic outcomes [57].

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Appendix

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Lutz Kockel, Kim Kerr, Michael Melnick and Norbert Perrimon. Insulin Signaling Circuitry in *Drosophila.* Manuscript in preparation (see appendix).

Meeting Abstracts:

Keystone Symposium: Diabetes Mellitus: Molecular mechanisms, Genetics and New Therapies. Keystone, Colorado, Jan 27th-Feb 2nd, 2005

Genome wide analysis of Akt phosphorylation by dsRNAi in *Drosophila*

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Insulin Receptor (InR) signaling is conserved in metazoans like *Drosophila* and humans. Roles of Insulin signaling include the control of cell proliferation, cell size, metabolic state and trophic survival.

We developed a quantitative cell based high throughput assay for *Drosophila* Akt activation termed Cytoblot / In cell Western: Drosophila KC_{167} cells are grown and RNAi treated in a 384 well plate, and plate-reader analyzed with a phospho-specific antibody against Ser^{505} (homologous to Ser^{473} of mammalian Akt) of dAkt. This assay allows us to analyze the response of Akt to various stimuli and perturbations.

We confirmed the validity of the assay by RNAi dependent knockdown of all known components of the dTor and InR pathway. RNAi knock-down of known upstream components required for Akt activation suppress Ser^{505} phosphorylation, while perturbations downstream of Akt (e.g. Rheb, dTor, S6K) result in a gain of phosphorylation, indicating the removal of feedback inhibition. Consistent with the RNAi approach, we observe similar effects when dTor is inhibited upon metabolic starvation and Rapamycin inhibition. We will present a biochemical and an *in vivo* analysis of the feedback circuitry.

In order to identify new components and regulators of dAkt, we performed genome-wide dsRNAi screens. We will present a comprehensive analysis of the Akt signaling network in *Drosophila.*

This is essentially the abstract I used without any major changes for the following conferences:

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Insulin signaling pathway circuitry in *Drosophila*

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Abstract

Activation of Akt by phosphorylation is a central step of insulin signal transduction in controlling cell proliferation, cell size and metabolism. Akt represents a nexus between Insulin Receptor (InR) and Tor signaling. Akt activity must be carefully balanced as ectopic activation is frequently associated with cancer, while non-sufficient Akt signaling is linked to metabolic disease and diabetes mellitus. To study the regulatory circuitry Akt is embedded in, we developed a cell based assay using a phospho-specific antibody against the hydrophobic motif site in *Drosophila* and screened a genome wide dsRNA library. Here, we show in cell culture that Akt is regulated by a negative feedback loop from S6K to InR. The negative feedback regulation is conserved *in vivo*. Further more, we present evidence of a second regulatory loop within the insulin signaling pathway connecting the two Tor containg complexes TORC1 and TORC2. This work portrays the InR-Akt and Tor-S6K signaling pathways as two branches of a highly connected, interdependent signal transduction network.

Introduction

The highly conserved insulin signal transduction pathway mediates growth factor and nutrient-dependent signaling from the extra-cellular space into the cell. In *Drosophila*

as well as in mammals, it controls cell proliferation and the size of cells, organs and animals. Furthermore, it acts as major anabolic switch of tissues and cells by controlling the uptake of carbohydrates and amino acid, inhibition of gluconeogenesis, and regulating translational activity (Rulifson et al., 2002; Teleman et al., 2005; Thomas, 2000).

The activity of the insulin signaling pathway has to be tightly regulated, as pathological activation of the pathway is associated with cancer, while insufficient activation results in morbidity by metabolic disease. This is achieved by an intricate arrangement of positive and negative regulators of pathway activity as well as regulatory feedback within the pathway itself (Manning, 2004). This design buffers at least in some cases the most deleterious effects caused by functional inactivation of individual genes of the pathway (Ma et al., 2005; Manning et al., 2005). Therefore, understanding the pathway topology opens novel routes for therapeutic intervention.

The *Drosophila* insulin signaling cascade consists of the orthologs of insulin (*Drosophila* insulin-like peptides, dIlps), the insulin Receptor (InR), the negative regulator Susi/B4, the insulin Receptor Substrate (Chico), the catalytic and adaptor subunits of Phosphatidylinositol 3-kinase (PI3K92E/Dp110 and PI3K21B/p60, respectively), the phosphoinositol (3,4,5) phosphate (PIP3) phosphatase Pten, the constituents of the *Drosophila* Tor complex 2 (TORC2), PDK1 and Akt (Leevers, 2005; Wittwer et al., 2005). With the exception of the dIlps, there is no known genetic redundancy within the insulin signaling pathway. Further, no IGF-type ligand or receptor is present in the *Drosophila* genome (Brogiolo et al., 2001).

Ligand-dependent stimulation of InR activates PI3K at the plasma membrane, which generates PIP3 by the phosphorylation of phosphoinositol (4,5) bisphosphate at the 3' position. Proteins like PDK1 and Akt are recruited to the plasma membrane via their pleckstrin homology domain which binds PIP3. Subsequently Akt is activated by a dual phosphorylation mechanism. The Akt activation loop within the kinase domain is phosphorylated by PDK1 (Brazil and Hemmings, 2001), while the C-terminal hydrophobic motif (mammalian Ser473, *Drosophila* Ser505) is phosphorylated by the TORC 2 complex (Sarbassov et al., 2005). This Rapamycin-insensitive multi-protein complex was first characterized in *S. cerevisiae*, and its composition is at least partially conserved in higher eukaryotes, with clear orthologs for TOR2 (Tor), AVO3 (Rictor), LST8 (mLst8/GβL) and Avo1 (Jacinto et al., 2004; Kim et al., 2003; Loewith et al., 2002; Sarbassov et al., 2004). In mammals, phosphorylation of Ser473 highly correlates with Akt kinase activity (Luo et al., 2003).

More recently, a second signal transduction pathway has emerged from a series of genetic studies in *Drosophila* connecting individual gene products controlling cell growth, some of them long known. The pathway consists of the orthologs of the Tsc1 and Tsc2 tumor suppressor complex (Gao and Pan, 2001; Potter et al., 2001; Tapon et al., 2001), the small GTPase Rheb (Saucedo et al., 2003; Stocker et al., 2003; Zhang et al., 2003), the components of the Tor complex 1 (TORC1) and *Drosophila* S6K (Montagne et al., 1999). Like TORC2, TORC1 was initially discovered in yeast and shares LST8/GβL and Tor as common components with TORC2 (Jacinto et al., 2004; Kim et al., 2003; Loewith et al., 2002). In contrast to TORC2, TORC1 activity is Rapamycin-sensitive and contains KOG1/Raptor as a unique constituent (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002). As in mammals, the *Drosophila* Tsc1/Tsc2 complex directly regulates the activity of Rheb via the GTPase activating protein (GAP) domain of Tsc2 (Garami et al., 2003; Inoki et al., 2002; Saucedo et al., 2003; Stocker et al., 2003; Tee et al., 2003; Zhang et al., 2003) while Rheb controls the activity of the Tor - Raptor complex (TORC1), presumably by directly binding to Tor (Long et al., 2005a; Long et al., 2005b; Tee et al., 2005). In mammals as well as *Drosophila*, Tor is strictly required

for the activation of S6K by means of direct phosphorylation of the S6K linker region. Similar to the insulin signaling pathway, the Tsc1/Tsc2 - Rheb - Tor - S6K pathway is highly conserved across phyla in terms of its constituents, the associated biochemical activities, their hierarchical organization and the associated physiological roles in cell growth and proliferation (Kwiatkowski, 2003; Manning and Cantley, 2003; Pan et al., 2004).

Both the Tsc1/Tsc2-Tor-S6K and insulin pathways have been recognized for regulating common phenomena like cell size and proliferation. More recently, the molecular connection of the two pathways has been pinpointed to direct Akt-mediated phosphorylation of Tsc2 on several sites (Dan et al., 2002; Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002). In cell culture phosphorylated Tsc2 appears to have reduced GAP activity and appears to be inhibited, resulting in reduced suppression of Rheb and upregulated Tor activity as measured by phosphorylation of the S6K linker region.

Despite the apparent linearity of the insulin signaling pathway, two feedback loops have been described within the pathway itself. The first one is mediated by transcriptional regulation of *InR* by the FOXO transcription factor (Junger et al., 2003; Puig et al., 2003). Active Akt directly phosphorylates and thereby inhibits FOXO, which results in a downregulation of *InR* transcripts, while inhibition of Akt results in transcriptional upregulation of *InR* (Junger et al., 2003; Puig et al., 2003). The second feedback loop has been extensively characterized in mammalian systems and is mediated by S6K dependent inhibition of IRS (Harrington et al., 2004; Shah et al., 2004; Um et al., 2004). Activation of S6K by mutation of either Tsc1 or Tsc2 leads to phosphorylation of IRS1 on several sites and cumulates in the proteolytic destruction of IRS1, resulting in insulin resistance (Harrington et al., 2004; Shah et al., 2004). Conversely, S6K1 knock-out mice are hypersensitive to insulin and show blunted IRS1 phosphorylation (Um et al., 2004).

Understanding the feedback circuitry holds high promise to find novel therapeutic targets to treat cancer and metabolic disease. Recent findings underline the non-reciprocal cooperativity of the *Pten* and *Tsc2* tumor suppressor genes based on the attenuated activity of Akt in tumors lacking Tsc2 (Ma et al., 2005; Manning et al., 2005). Moreover, the lean phenotype and insulin hypersensitivity of mice lacking S6K1 show a high potential of the Tor-S6K signaling branch as point of therapeutic intervention against type 2 diabetes and obesity as suggested by Um and colleagues (2004). Interestingly, Tor activity is regulated by amino acid availability, providing a molecular framework to understand the effect of starvation as the earliest treatment of diabetes (Dean, 2004).

Here, we study the general outline and feedback circuitry of the *Drosophila* InR signal transduction pathway by dsRNA-mediated interference in a tissue culture model and in the developing 3rd instar wing imaginal disc. We find that the general outline of the S6K dependent inhibitory feedback loop is conserved in *Drosophila*, but the target of inhibition is InR and not the IRS ortholog Chico. Moreover, our data suggests the presence of an additional regulatory loop involving the equilibrium of the two Tor containing complexes TORC1 and TORC2. This work portrays the InR-PI3K-Akt and Tsc1/Tsc2- Tor-S6K signaling pathways as two branches of a highly connected, interdependent signal transduction network, showing that unbiased RNAi screens are an excellent tool for gaining further insight into the mechanism of *Drosophila* insulin signaling.

Results

An assay for *Drosophila* phospho-Akt

To establish a cell-based assay for regulators of insulin signaling in *Drosophila* that could be used in a genome-wide RNAi screen, we tested 64 commercially available phospho-antibodies generated against conserved mammalian proteins of the insulin signal transduction pathway by western blot and immunofluorescence on *Drosophila* Kc_{167} cells (data not shown). While approximately 10% of the antibodies tested cross-reacted on Western blots in an insulin-dependent manner, none of them recognized an insulin induced antigen on Kc_{167} cells. Thus, to generate a robust antibody-based readout tool for insulin signaling, we generated a phosphospecific antiserum recognizing the phosphorylation of the C-terminal hydrophobic motif of *Drosophila* Akt, Serine 505 or Ser 586 of the short and long splice form, respectively (hereafter referred to as anti P-Akt). Phosphorylation of the hydrophobic motif closely reflects activity of Akt (Luo et al., 2003). Stimulation of Kc_{167} cells with insulin for 10 minutes induced a robust P-Akt signal, which was strongly suppressed when known components of the insulin signaling cascade like InR, Chico, the catalytic subunit of PI3-Kinase PI3K92E and Akt itself were silenced by RNAi (Figure 1A-E'). We next asked whether the anti P-Akt antibody detected differences in Akt phosphorylation in the 3rd instar imaginal disc induced by expression of dominant negative insulin receptor (InR^{DN}) or a constitutively active catalytic subunit of PI3K ($PI3K^{CAAX}$) using the UAS-Gal4 expression system (Brand and Perrimon, 1993). Staining of *wt* imaginal wing discs revealed no pattern of P-Akt associated with compartments or compartment boundaries (not shown). We used apterous-Gal4 (ap-Gal4) to drive expression of Inr^{DN} and $PI3K^{CAAX}$ concomitant with membrane tagged GFP in the dorsal compartment and compared levels of P-Akt to ventral cells as controls (Figure 1F-G'). As expected, expression on InR^{DN} resulted in a reduction of P-Akt (Figure 1F and F') and $PI3K^{CAAX}$ expression drastically increased the levels of P-Akt when compared to ventral control cells (Figure 1G and G'). Western blotting experiments of extracts from Kc_{167} cells pretreated with dsRNAs against InR, PI3K92E, and Akt confirmed the specificity found in the immunohistochemical stainings on cells and *Drosophila* tissue (Figure 1H). Surprisingly, RNAi against PDK1, the Akt Ser308 kinase ortholog in *Drosophila*, enhanced P-Akt Ser505 phosphorylation rather than suppressing it. However, this finding is consistent with mammalian tissue culture results (Hsieh et al., 2004; Williams et al., 2000) (see discussion). Taken together, our data show that anti-PAkt faithfully detects Akt phosphorylation in cell culture and *in vivo*.

Genome-wide RNAi screen for regulators of P-Akt

In order to search for components of the insulin signal transduction pathway and to identify novel regulatory inputs, we used the Cytobloting/In Cell Western method (Figure 2A) as a fast and quantitative cell-based highthroughput assay. Cells were grown in 384 well plates and, after three days in the presence of various dsRNAs, were fixed and immunostained with anti-P-Akt antiserum. Bound primary antibody was quantified by near Infra-Red dye conjugated secondary antibody in a 384 well plate reader. Values were normalized to total cell counts as measured by fluorescent nuclear staining.

Genome-wide RNAi screens for modulators of Akt phosphorylation were done in replicates without stimulation (to determine baseline levels) and after 10 min. of insulin stimulation. Employing a cutoff value of +/- 2 Standard Deviations (SDs) relative to the screen average, we found that 126 dsRNAs resulted in the suppression of Akt phosphorylation while 172 enhanced it. Importantly, we identified 9 out of the 11 known components upstream of Akt, validating the reliability of this method (Figures 2B and C). PDK1 RNAi resulted in an upregulation of P-Akt Ser505, consistent with the results shown in Figure 1H. The two known regulators not to be found in the screens were Chico and Pten, which pass the cutoff threshold. Here we focus on an analysis of the interaction

between the previously known components of the pathway. A detailed analysis of novel components identified in the screen will be reported elsewhere.

Surprisingly, we found that Akt phosphorylation is sensitive to the interference of the Tsc1/Tsc2-Tor-S6K signaling branch (Figures 2B and 2C), classically viewed as downstream components of Akt (Kwiatkowski, 2003; Manning and Cantley, 2003; Pan et al., 2004), which seems counter-intuitive as phosphorylation of Akt should reflect events regulated by upstream elements of the signal transduction pathway but not by downstream regulators. dsRNAs against downstream mediators required positively for insulin signal transduction like the small GTPase *Rheb*, the TORC1 component *Raptor* and *S6K* induced ectopic phosphorylation (activation) of Akt in the absence of an insulin stimulus. Conversely, dsRNAs against the negative regulators *Tsc1* and *Tsc2* suppressed the P-Akt signal when the pathway was in its active (insulin stimulated) state. Altogether, these results suggest the existence of an inhibitory feedback signal mediated by the components downstream of Akt, namely Rheb, Rictor, Tsc1/2 and S6K. Accordingly, when the negative feedback is disrupted, ectopic activation of Akt is triggered.

Feedback regulation of Akt phosphorylation by the Tsc1/Tsc2-Tor-S6K signaling branch

To confirm the results of the genome-wide RNAi screens, we re-synthesized independent dsRNAs against most of the known pathway members and retested their effects on P-Akt in seven replicates by cytoblot using an external standard for normalization against cell number dependent effects (Figures 3A and B, see Experimental Procedures). dsRNAs against *GFP*, *MEKK1/4*, *thread* and *CSK* were used as negative controls, and *Pten* dsRNA as positive control. As found in the genome-wide screens, removal of the negative regulators Tsc1 and Tsc2 resulted in a suppression of P-Akt in presence of insulin, while knock-down of S6K elevated P-Akt at baseline conditions. This demonstrated that disrupting the feedback loop is most effective under a non-stimulated condition.

In order to test the feedback hypothesis by different means than RNAi, we inhibited the activator of S6K, TOR, by two independent strategies (Figure 3C). First, in a chemical approach, we exposed cultured cells to the small molecule Rapamycin, an effective inhibitor of TOR in *Drosophila* (Hidalgo and Rowinsky, 2000; Mita et al., 2003; Sawyers, 2003). Second, in a metabolic approach, we starved cultured cells in amino acid free media, thereby potently inhibiting TOR activity (Gao et al., 2002; Radimerski et al., 2002a). Amino acid starvation, as well as Rapamycin-induced TOR inhibition, led to a highly significant increase in P-Akt compared to control cells treated with amino acid-containing medium or methanol solvent control, respectively. This confirmed the RNAi data and validates the idea of a negative feedback loop auto-regulating the activity of the pathway after stimulation.

We next asked whether the inhibitory potential of S6K correlates with its activity, using the phosphorylation of *Drosophila* S6K linker region Serine 398 (homologous to mammalian S6K1 Ser389) as readout (Figure 4). Total lysates of *Drosophila* Kc167 cells pretreated with dsRNAs against *Tsc2*, *Raptor*, *S6K* and *Rheb* were analyzed for S6K and Akt phosphorylation. Cells treated with dsRNAs against luciferase and non-RNAi treated cells served as negative controls (Figure 4A, lanes 5 and 6). Enhancement of P-Akt strongly correlated with suppression of the phosphorylation status of S6K, with a clear elevation of Akt phosphorylation when Rheb, Rictor or S6K expression was knocked down by RNAi.

In order to address how S6K mediates its feedback inhibition to Akt, we triggered ectopic Akt phosphorylation by RNAi against S6K and asked whether other components of the insulin signaling pathway were required for this effect by removing them in

addition to S6K (Figure 4B). Removal of S6K resulted in a robust enhancement of P-Akt by 4 SDs above non-RNAi treated control cells. Additional RNAi against control genes like *GFP*, *CSK* or *MEKK1/4* did not significantly change the P-Akt value, confirming that combined RNAi against two genes does not influence the efficiency of the RNAi silencing. As positive controls, co-RNAi of S6K and Pten resulted in an elevation of P-Akt levels, while dsRNA treatment against S6K and Akt reduced P-Akt to levels of unstimulated, non-RNAi treated cells. Interestingly, RNAi against the positive regulators InR and PI3K, did suppress the S6K RNAi induced ectopic Akt phosphorylation, indicating that the S6K-dependent feedback inhibition is mediated by these two components. However, co-RNAi of Chico with S6K did not reduce P-Akt. This is in contrast to recent reports in mammals showing S6K-dependent regulation of IRS transcription, phosphorylation and protein stability (Harrington et al., 2004; Shah et al., 2004; Um et al., 2004). Our results suggest that the feedback regulation is routed through InR and PI3K in a Chico-independent manner.

A S6K-independent TORC1 and TORC2 interconnection

One of the most striking results emanating from the analysis of the non-stimulated genome-wide P-Akt RNAi screen is that *Raptor* dsRNA exhibit a more than two-fold higher P-Akt value than S6K (Figure 2B). As the negative feedback loop is mediated through S6K, and dsRNA against S6K is very efficient (see Figure 4A, lane 3 and Figure 5E, lane 7), this effect cannot be simply explained by an exclusive involvement of Raptor in the S6K-dependent regulation of P-Akt. To confirm this result, we performed anti P-Akt immunohistochemical staining on Kc_{167} cells treated with dsRNAs against luciferase (negative control), Pten (positive control), S6K and Raptor (Figure 5 A-D). Confocal imaging and digital processing using identical settings confirmed Raptor RNAi triggering the highest levels of ectopic Akt phosphorylation when compared to S6K, Pten and luciferease. This result suggests that Raptor participates in an additional regulatory loop interconnecting the InR-PI3K-Akt and Tsc1/Tsc2-Tor-S6K branches.

We therefore tested the phosphorylation status of Akt and S6K in the presence or absence of functional TORC1 and TORC2 (Figure 5E). Both complexes contain Tor and GbL as common components. In addition, TORC1, responsible for S6K Ser398 phosphorylation (Oldham and Hafen, 2003; Radimerski et al., 2002a), contains the unique component Raptor. Further, Rictor and Avo1 are unique to TORC2, the Akt Ser505 kinase (Sarbassov et al., 2005). As shown above (Figure 4), the phosphorylation of Akt is elevated when the expression of Raptor is silenced by RNAi, while P-Akt is strongly suppressed when Rictor and Avo1 are removed. Conversely, the phosphorylation of S6K is strongly reduced when cells are treated with *Raptor* and *Tor* dsRNAs. Interestingly, phospho-S6K levels are elevated when TORC2 function is disrupted by RNAi against Avo1 and Rictor, however to the same extent as seen in the case of P-Akt and Raptor RNAi. We interpret the result in favor of a regulatory connection between TORC1 and TORC2, where disruption of one complex elevates the activity levels of the other.

Feedback regulation of P-Akt in the *Drosophila* wing imaginal disc

While our data clearly indicated regulation of P-Akt in *Drosophila* Kc_{167} cells by a feedback loop, we wanted to test whether this mechanism is also conserved *in vivo*. This is particularly important since previous experiments have shown that some of the connections within the insulin signaling pathway observed in *Drosophila* tissue culture cells are either not physiological relevant or are absent in the whole organism (Dong and Pan, 2004; Potter et al., 2002; Radimerski et al., 2002b). In order to test if P-Akt is regulated by the Tsc1/Tsc2-Tor-S6K signaling branch *in vivo*, we used the UAS-Gal4

system (Brand and Perrimon, 1993) to co-express Tsc1 and Tsc2 in the dorsal compartment of the third instar imaginal wing disc under the control of ap-Gal4 (Figure 6). Co-expression of both Tsc1 and Tsc2 has been shown previously to strongly inhibit TORC1 and S6K activity, leading to reduced cell and organ size (Gao and Pan, 2001; Potter et al., 2001; Radimerski et al., 2002a; Tapon et al., 2001). Therefore, if the feedback loop was intact *in vivo*, we expected an upregulation of P-Akt when compared to ventral control cells. This is indeed what we observed - a clear increase of P-Akt in Tsc1, Tsc2 overexpressing cells (Figure 6A'). To ask the converse question if P-Akt levels were reduced when Tsc1/Tsc2 function is reduced, we induced homozygous mutant clones of $tsc1^{Q87X}$, a null allele, in the wing disc and analyzed Akt phosphorylation by staining with anti P-Akt. Mutations in either *Tsc1* or *Tsc2* result in loss of function of the Tsc1/Tsc2 tumor suppressor complex and should therefore result in the converse P-Akt phenotype than the overexpression experiment. We found reduced P-Akt levels in $tsc1^{Q87X}$ mutant tissue when compared to *wt* control tissue (Figure 6B'). In conjunction with the Tsc1/Tsc2 overexpression data this confirmed that P-Akt is regulated by feedback signaling *in vivo*.

We next asked whether Tor was required for Akt Ser 505 phsophorylation *in vivo*. Our tissue culture Western blot results detected only a mild effect of Tor RNAi on Akt phosphorylation, possibly because the antagonizing effects of removing feedback inhibition concomitant with TORC2 disruption in a background of partial RNAi knockdown. Therefore, we induced $tor^{\Delta P}$ homozygous mutant clones in a *wt* as well as $Pten^{DJ189}$ mutant background (Oldham and Hafen, 2003). It was noted before that loss of Tor function suppresses the loss of Pten (Oldham and Hafen, 2003). We found that P-Akt is upregulated in $Pten^{Dj189}$ loss of function clones (Figure 7A and A'). This elevation of P-Akt was suppressed when *Tor* function is removed simultaneously with $Pten^{DJ189}$ (Figure 7B and B'). Clones of cells homozygous for the $Tor^{\Delta P}$ allele showed a suppression of P-Akt in a *wt* background, however we observed this reduction only in clones that were induced early during larval life (see Experimental Procedures). Altogether, we conclude that Akt phosphorylation is dependent on Tor function *in vivo* as expected **(Ref Sabrassov)** and importantly that the regulatory feedback of Akt by the Tsc1/Tsc2-Tor-S6K signaling branch is conserved in *vivo*.

Discussion

Dissecting the insulin signaling circuitry using RNAi

Our RNAi-based analysis of the insulin pathway has led us to gain a number of insights on the topology of the InR-PI3K-Akt and Tsc1/Tsc2-Tor-S6K signaling branches and the dual phosphorylation mechanism of Akt activation.

PDK1, although an activating kinase of Akt, was identified in our screen as a suppressor of P-Akt, a finding consistent with the literature (Hsieh et al., 2004; Williams et al., 2000). This observation can be rationalized in two ways. First, Akt protein expression is upregulated in *PDK1* RNAi-treated cells (Figure 1H). Second, PDK1 phosphorylation is targeted at the kinase domain, a site not recognized by the anti-P-Akt antibody directed against the hydrophobic motif used in this study. However, removal of PDK1 by RNAi gives rise to inactive Akt, which leads to inactive S6K (Figure 5E) and the disruption of the negative feedback inhibition to the upstream part of InR signaling. Therefore, the upregulation of phosphorylation of the hydrophobic motif is a logical consequence of PDK1 expression silencing. Thus, the classification of PDK1 as a suppressor is - although misleading - in accordance with the signaling circuitry displayed in Figure 8.

From our screening effort, depletion by RNAi of Rictor and Avo1 lead to strong suppression of insulin-induced phosphorylation of Akt, consistent with its described role as TORC2 component, the Akt hydrophobic motif kinase (Sarbassov et al., 2005). Components like Tor and GβL, constituents of both TORC1 and TORC2, come out of the screen as molecules required for Akt Ser505 phosphorylation, but with a clear low score. Why do the shared TORC components score at lower strength? RNAi against Tor and GβL have dual, antagonistic effects: In addition to reducing the TORC2 activity towards Akt, it weakens the TORC1 complex, silence S6K activity and therefore remove feedback inhibition. In flies, only Tor clones induced very early in larval development show a reduction of endogenous P-Akt levels. A long protein half life of *Drosophila* Tor would account for this observation. In addition, only minimal levels of functional TORC2 complex might be sufficient for Akt Ser505 phosphorylation, presumably at the plasma membrane **(Sabrassov)**, while higher levels of TORC1 are required for S6K phosphorylation, making this complex more sensitive to RNAi-induced disruption (Figure 5).

Regulation of Akt phosphorylation *in vivo*

Our initial experiments on wing imaginal disc tissue expressing either activated PI3K or dominant negative InR displayed very high activation by $PI3K^{CAAX}$ expression, but a comparatively low suppression of P-Akt by InR^{DN} (Figures 1F-G'). We interpret this finding as a relatively low constitutive phosphorylation of Akt present in the 3rd instar imaginal wing disc, which has much more "headroom" for activation than for suppression. In agreement with this hypothesis, the relative upregulation of P-Akt overexpressing Tsc1 and Tsc2 is clearly higher than the suppression of P-Akt in $tsc1^{Q87X}$ homozygous mutant clones. However, we cannot exclude that some unspecific background binding of the anti P-Akt antiserum contributes to the observed effect. In addition, our analysis provides only a snapshot into the developmental dynamics of Akt phosphorylation, and P-Akt levels might be higher at different times of development.

The elevated P-Akt levels of the Tsc1-Tsc2 co-expression experiment is a result clearly in favor of a conserved negative feedback regulation of Akt by the Tsc1/Tsc2 tumor suppressor complex *in vivo*. However, it is surprising at two levels. First, Akt has been described as regulator of cell size, and akt^{1} homozygous mutant clones show reduced cell size. However, the outcome of Tsc1/Tsc2 co-expression is reduced cell size, despite the elevated levels of P-Akt (Gao and Pan, 2001; Potter et al., 2001; Tapon et al., 2001). The converse experiment highlights the same paradox: $tsc1^{Q87X}$ mutant cells have a larger cell size, despite lowered P-Akt levels (and activity). These results might indicate that for cell size, the only function of Akt is the regulation of Tsc1/Tsc2 activity. In support of this premise, no other Akt substrate (e.g. FOXO, Gsk3β) has been shown to elicit a cell size defect so far (Junger et al.).

The second surprise lays in the fact that Tsc1/Tsc2 overexpression triggers ectopic Akt activation, which should however inactivate Tsc2 by phosphorylation and therefore lower the P-Akt phosphorylation status. If the Akt-Tsc2 link is of less physiological relevance as initially suggested (Dong and Pan, 2004; Potter et al., 2002), or if overexpressed Tsc1/Tsc2 localizes to a subcellular compartment where it escapes phosphorylation by active Akt but can elicit Rheb inhibition remains to be seen.

The TORC1-TORC2 connections

A centered view on the two Tor containing complexes TORC1 and TORC2 reveals a number of connections between the InR-PI3K-Akt and Tsc1/Tsc2-Tor-S6K branch. They are, at least in tissue culture systems and in some pathological states like

cancer, molecularly closely connected by the direct phosphorylation of Tsc2 (a TORC1 regulator) by Akt (a TORC2 substrate) (Dan et al., 2002; Inoki et al., 2002; Ma et al., 2005; Manning et al., 2005; Manning et al., 2002; Potter et al., 2002; Sarbassov et al., 2005). They share a second link comprised of S6K (a TORC2 substrate) negatively feeding back on the activity of the upstream part of the InR signaling pathway required for Akt activation (Harrington et al., 2004; Radimerski et al., 2002a; Shah et al., 2004; Um et al., 2004). Our data suggest the presence of a third link, where disruption of one TORC complex increases the activity of the other. In a strict sense, this can be interpreted as mutual inhibition of TORC1 and TORC2, suggesting an assembly/disassembly equilibrium between TORC1 and TORC2 as a mechanism, possibly driven by mass action law (Figure 8). Recent work in *S. cerevisiae* has shown that TORC2 disintegrates when cells are depleted from Avo1 or Avo3 (Rictor). Thus, if the requirements of Avo1 and Rictor for TOR complex 2 integrity are conserved in higher eukaryotes, Tor/GβL would be free to be recruited into TORC1 complexes.

On initial inspection, the effect of Raptor removal on Akt phosphorylation seems to be much more drastic than the effect of removing Rictor on P-S6K levels (Figure 5E). However, the amplitude of P-Akt level changes upon depletion of Raptor is bigger because the Akt feedback inhibition is disrupted in addition to any putative shifts in the TORC1-TORC2 equilibrium. The relative differences of P-Akt levels from S6K to Raptor RNAi cells compared to changes of P-S6K in luciferase versus Rictor or Avo1 RNAi cells reveal a much more comparable effect. Nevertheless, the reciprocity of the TORC1-TORC2 equilibrium remains to be quantified.

The TORC2 complex has been suggested as an attractive therapeutic target (Bellacosa et al., 2005; Sarbassov et al., 2005). However, the tight interconnection of TORC1 and TORC2 warrants caution as inhibition of one complex may lead to the activation of the other one.

Experimental Procedures

Cell culture and RNAi in *Drosophila* Kc_{167} cells. For 384 well plate experiments cells were uniformly and rapidly dispensed into clear bottom black 384 well plates (Corning) containing 250ng of individual, arrayed dsRNAs using a MultiDrop (Thermo) liquid dispenser at 8 x 10^3 cells per well in 10 µl of serum free media per well. After 60 min incubation, 70 µl of 10% serum-containing culture medium (Schneider's Medium, Invitrogen) per well was added. After three days incubation at 25C, cells were washed once and starved in 80 µl serum free medium overnight (12 hrs). For insulin stimulation, cells were exposed to 387 nM bovine insulin (Sigma) for 10 min. Rapamycin was used at a final concentration of 50 nM for 4 hrs, amino acid free media (Atlanta Biologicals) was used for 8 hrs. For six-well dish experiments for Western blotting all the conditions were chosen similarly as described above, with 10 µg of dsRNA being added to 1.5 x 10^6 cells per well in 1 ml of Serum free media, supplemented after 60 min with 5.ml of Serum containing media. For eight-well chamber slides for immunofluorescence, cells were treated as described above, using 2 µg of dsRNA per well in 100 µl of non-Serum containing media, complemented with 500 µl of Serum containing media after 60 min.

Cytoblot. Tissue culture medium was removed and cell were fixed with 6% Formaldehyde in PBS for 10 minutes (40 µl per well), permeabilized in PBS containing 0.1% Triton X-100 for 30 minutes (40 µl per well) and blocked in 5% non-fat milk in PBS for 60 minutes (90 µl per well). 20 µl of anti-*Drosophila* P-Akt Ser505 primary antibody (Cell Signaling Technology, Beverley, MA) was added per well and incubated at 4C overnight (1:800 diluted in 5% non-fat milk). After 3 washes with PBS (80 µl per well), 20 µl secondary antibody (goat anti-Rabbit AlexaFluor 680 diluted 1:2,500, Invitrogen ("second generation Cytoblot") or goat anti-Rabbit HRP ("first generation Cytoblot"), Jackson Laboratories, 1:1,200) was added in 5% non-fat milk and incubated for 90 minutes. DNA stain was performed with Sytox Green (Invitrogen) 1:20,000 in PBS for 30 min (40 µl per well). After 3 additional washes with PBS, plates were filled with 20 µl PBS per well. The fluorescent value of the Sytox dye DNA stain, referred hereafter to as nuclear fluorescence (NucFl), is interpreted as the value representing relative cell numbers per well and were read in a Molecular Devices plate reader (520/560 nm). AlexaFluor 680 fluorescently stained plates (interpreted as value representing relative Phospho-Akt amounts per well) were read in a LiCor Aerius plate reader (680/720 nm). The PBS of the HRP-conjugated secondary antibody stained plates was removed and replaced by 20 µl SuperSignal West Pico Chemiluminescent Substrate (Pierce) and read with Luminescent settings on the Molecular Devices plate reader (also interpreted as value representing relative P-Akt amounts per well). All liquid manipulation steps were performed using a MultiDrop.

Data analysis. All individual values quantifying amounts of Phospho-Akt were normalized to the cell number per well using the nuclear fluorescent value from the DNA stain. Robust linear regression was performed on the log2(P-Akt/NucFl) values of each individual screen plate, and residuals from the log2(P-Akt/NucFl) values to the regression line were calculated. All residuals of each genome-wide screen were pooled and a cell number dependent error model was developed and used to determine locally weighted standard deviations (SD) and averages in dependence of cell number. Z-Scores using these two parameters were calculated, expressing the deviation from the local average value in SDs. All Z-Scores were corrected against position effects by setting the Mean Z-Score of each individual well position across one genome-wide screen replicate to zero.

The details of the genome-wide RNAi screen and its analysis will be published elsewhere (L. Kockel, K. Kerr and N. Perrimon, in preparation). For non-genome-wide RNAi experiments, an external standard consisting of 768 values of non-RNAi treated cells covering the whole spectrum of cell densities was used to determine cell number dependent averages and SDs to calculate experimental Z-Scores of RNAi treated wells. All P-Akt values of non-stimulated cells are normalized using a baseline standard curve (the average non-treated, non-stimulated experiment scores zero). For the insulin-stimulated case, the P-Akt values of insulin-treated cells are normalized using a standard curve derived from insulin-stimulated cells (the average non-treated, insulin-stimulated experiment scores zero).

Antibodies. All P-Akt indirect immunofluorescence images, Cytoblots and Western blots were performed using anti-*Drosophila* P-Akt Ser505 using a 1:200, 1:800 and 1:200 dilution, respectively. For immunofluorescence, AlexaFluor594 and AlexaFluor488 conjugated secondary antibodies against Rabbit and Goat were used (1:500, Invitrogen). Western blotting was performed using HRP conjugated anti-rabbit and anti-mouse antisera (Amersham). Pan-Akt and P-S6K Ser398 (Cell Signaling Technology, Beverley, MA) was used 1:200. Anti-GFP was purchased from Cappel and used 1:4000. Mouse anti α-Tubulin (Sigma) was used 1:500 and 1:2000 for immunofluorescence and Western blotting, respectively. Rabbit anti dS6K was a generous gift from Marry Steward and used 1:10,000 for western blotting.

Immunofluorescence. Imaginal discs and *Drosophila* Kc_{167} cells were fixed using 6% Formaldyhyde in PBS (cells 10 min at room temperature, imaginal discs at 4 C^0 over night), permeabilized in 0.1% Triton X-100 (2 hrs) and blocked with 5% BSA in PBS (1 hr). Primary antibody incubation in 5% BSA was performed overnight at 4 C^0 with antibody dilutions as indicated above. After 3 washes with PBS, secondary antibody was incubated over night in 5% BSA, followed by 3 washes in PBS. Specimens were mounted using Vectrashield mounting medium with DAPI (Vector Co.).

Genetics. Mutant wing imaginal disc clones were generated by FLP/FRT-mediated mitotic recombination using the following chromosomes: *FRT82, Tsc1^{Q87X}* (Tapon et al., 2001). *FRT40A, Pten^{dj189}*, *FRT40A, Tor$^{\Delta P}$*, *FRT40A, Tor$^{\Delta P}$*, *Pten^{dj189}*. Males of the respective genotypes were crossed to *y,w,hs-FLP, UAS-mCD8::GFP; FRT40A,tub-Gal80; tub-Gal4/TM6B* or *y,w,hs-FLP,UAS-mCD8::GFP; tub-Gal4; FRT82B,tub-Gal80/TM6B* females and larvae were heat shocked 60 hrs +/- 12 hrs after egg laying (unless otherwise specified) at 37C for 15 min. Overexpression of PI3K^{CAAX} (Leevers et al., 1996), InR^{DN} (Bloomington *Drosophila* Stock Center), Tsc1 and Tsc2 (Tapon et al., 2001) in the dorsal compartment of the wing imaginal disc was performed using the Gal4-UAS system (Brand and Perrimon, 1993) with *y,w; ap-Gal4,UAS-mCD8::GFP* (gift from C. Micchelli).

Gene names. Based on BLAST searches, information in the public ortholog databases InParanoid (O'Brien et al., 2005), and Homologene (Wheeler et al., 2005) published sequence homologies (Kim et al., 2003), CG3004 (Fbgn0030142), and CG10105 (0033935) are referred to as GβL and Avo1, respectively.

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Figure legends

Figure 1. Specificity of the anti P-Akt (Ser505) antibody. (A-E') *Drosophila* Kc_{167} cells stained with DAPI (blue) anti P-Akt (Ser505) antibody (green) and anti α-tububin (red) at baseline (A'-E') or 10 min of Insulin stimulation (A-E). Cells were RNAi-treated as described in Experimental Procedures using no dsRNA (A & A'), *InR* dsRNA (B & B'), *chico* dsRNA (the IRS ortholog, C & C'), *PI3K93E* dsRNA (the caltalytic subunit of the class I *PI3-Kinase*, D & D') and *Akt* (E & E'). Please note that large polynucleated cells are resistant to the Insulin stimulus (A). (F-G') Close up views of single tangential optical sections on the dorso-ventral boundary at the wing pouch of 3rd instar wing imaginal discs, dorsal to the right. Wing discs are stained with DAPI (blue), anti P-Akt (Ser505) antibody (red) and anti-GFP (green), marking the expression domain of *apt-Gal4* and the *UAS-InR^{DN}* (F, F') and *UAS-PI3K^{CAAX}* (H, H') expression constructs. F' and H' show DAPI and P-Akt channels only, the compartment boundary is marked by a white line. (H) Western blot of crude extracts prepared from *Drosophila* Kc_{167} cells at base line (lanes 1-6) or Insulin stimulation (lanes 7-12) treated with dsRNAs as indicated.

Figure 2. Genome-wide screen for regulators of Akt (Ser505) phosphorylation. (A) Cartoon of the cytoblot technique used to screen 58 384 well plates containing dsRNAs covering the entire *Drosophila* genome in duplicates. Experimental values for Akt phosphorylation are normalized to the individual cell numbers per well determined by the DNA dye staining. See experimental procedures for details. (B, C) Ranked Z-Scores of genome-wide RNAi screens at baseline (B) and Insulin stimulation (C) with the known components of InR and Tor signaling marked in red.

Figure 3. Phosphorylation of Akt is regulated by the activity of the InR-PI3K as well as Tsc1/Tsc2-Tor signaling branch. (A, B) Calculated Z-Scored of Akt (Ser505) phosphorylation for independently synthesized dsRNAs against the InR-PI3K and Tsc1/Tsc2-Tor signaling pathway branches under baseline (A) or Insulin-stimulated (B) conditions. (C) Cytoblot derived calculated Z-Scores of P-Akt (Ser505) after Insulin stimulation of Kc_{167} cells pretreated with 50 nM Rapamycin (4 hrs) or amino acid (aa) and Serum free tissue culture medium (8 hrs). Methanol (MeOH) was used as solvent control. Each bar represents a single well of a multiple-replicate experiment. Experiments were analyzed using external standard curves as described in experimental procedures. *P-values* were calculated using the two-tailed students *t-test* assuming equal variance.

Figure 4. Inhibition of dS6K results in derepression of Akt by inhibition of InR. (A) Immunoblotting of crude lysates prepared from *Drosophila* Kc_{167} cells after 10 min of Insulin stimulation treated with dsRNAs as indicated. Non-RNAi -reated and luciferase (luc) dsRNA treated cells were used as negative controls. (B) Calculated Z-cores of P-Akt (Ser505) under non-stimulated condition derived from a cytoblot using dsRNAs as indicated. GFP, CSK and MEKK are used as negative controls, Pten and Akt as positive controls. Each value and its SD is derived from seven replicates.

Figure 5. Reciprocal regulation of dS6K phosphorylation by TORC1 and TORC2. (A) Single confocal section of dS6K, (B) Raptor, (C) Luciferase and (D) Pten RNAi-treated *Drosophila* Kc_{167} cells stained with DAPI (blue) P-Akt (Ser505) antibody (green) after 10 minutes of Insulin stimulation. Images were recorded and processed using identical conditions. Note the highest level of P-Akt (Ser505) in the Rictor RNAi-treated cells. (B)

Western blot of crude extracts prepared from Insulin-stimulated *Drosophila* Kc_{167} cells treated with dsRNAs against TORC1 (Raptor, lane1), TORC2 (Rictor, Avo1, lanes 4, 5) and components present in both complexes (GbL, Tor lanes 2, 3). dsRNAs against Akt, dS6K and Luciferase were used as controls.

Figure 6. Feedback regulation of Akt in the developing wing imaginal disc. (A-B') Single tangential optical sections of 3rd instar wing imaginal discs. Wing imaginal discs are stained with DAPI (blue), P-Akt (Ser505) antibody (red) and anti-GFP (green). (A, A') A magnified view on the dorso-ventral boundary at the wing pouch is shown. GFP expression (green) marks the expression domain of *apt-Gal4* driver and the *UAS-Tsc1, UAS-Tsc2* expression constructs. (B, B') $Tsc1^{Q87X}$ homozygous mutant clones located at the wing primordium. Mutant clones are marked by the expression of GFP (green). A' and B' show DAPI and P-Akt channels only, the compartment boundary (A') or the line tracing of the clones (B') is marked with by a white line.

Figure 7. Tor is required for Akt Ser505 phosphorylation in vivo. (A-C') Tangential confocal images of the wing pouch area of 3rd instar wing imaginal discs stained with DAPI (blue), P-Akt (Ser505) antibody (red) and anti-GFP (green). Mutant clones are marked by the expression of GFP (green). Homozygous mutant clones of (A,A') $Pten^{dj189}$, (B, B') $Pten^{dj189}$, $Tor^{\Delta P}$ and (C, C') $Tor^{\Delta P}$ located at the wing primordium. $Tor^{\Delta P}$ homozygous mutant clones were induced 36 hrs after egg laying. A'- C' show DAPI and P-Akt channels only, line tracing of the clones is marked with by a white line.

Figure 8. Model of the InR-PI3K-Akt and Tsc1/Tsc2-Tor-S6K signaling circuitry in *Drosophila*. dS6K elicits feedback inhibition via InR, resulting in Insulin resistance when highly active. *Drosophila* TORC1 and TORC2 complexes are connected by containing common components like Tor and GbL. In addition, disassembly of one TORC results in higher activity for the other, probably by a mass action equilibrium.

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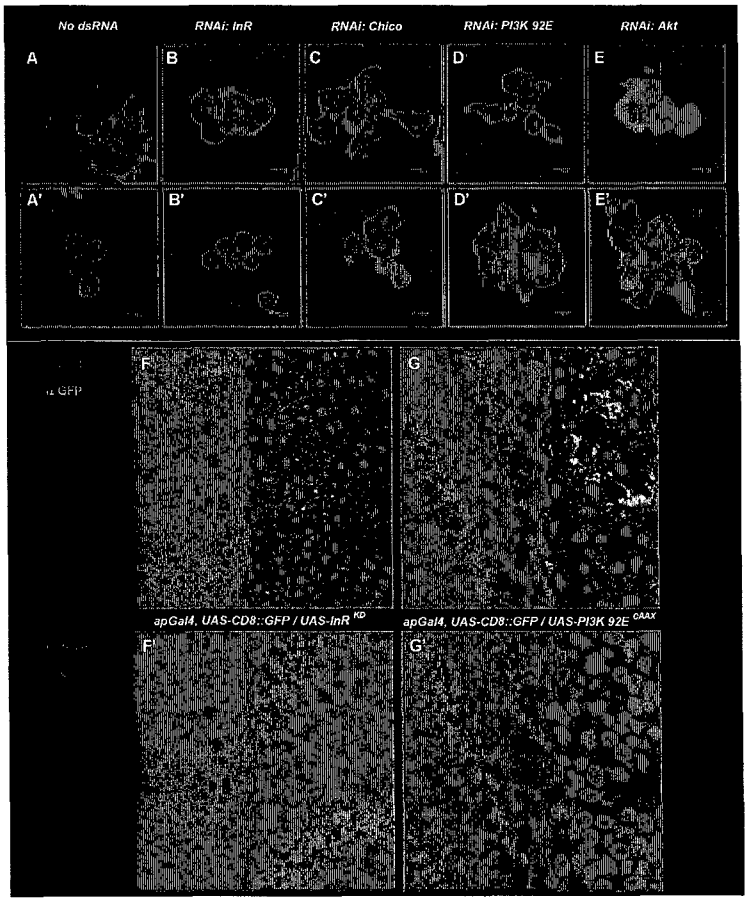
Figures

Figure 1



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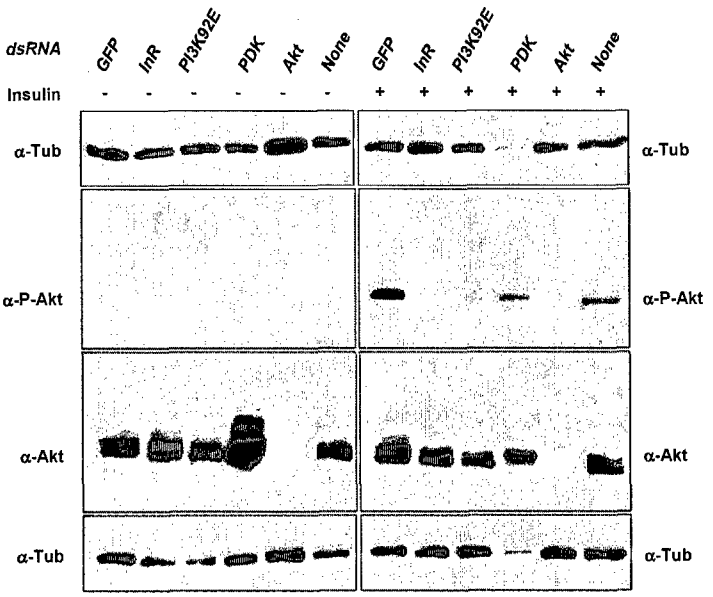
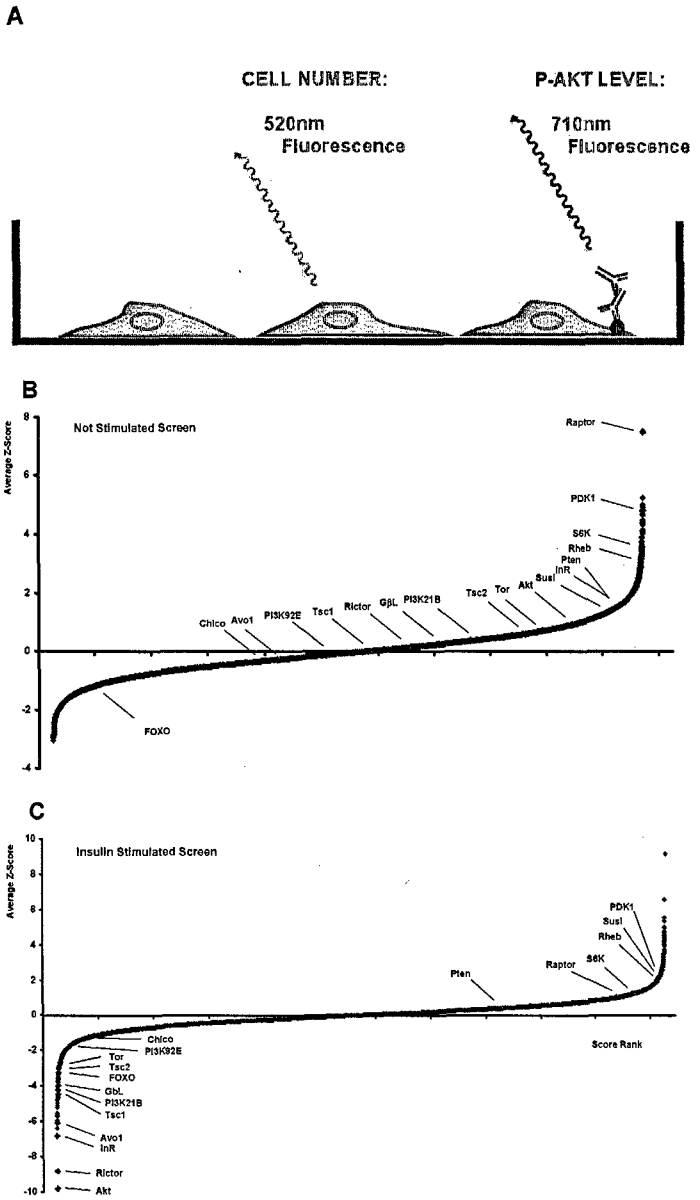


Figure 2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Akt

Figure 3

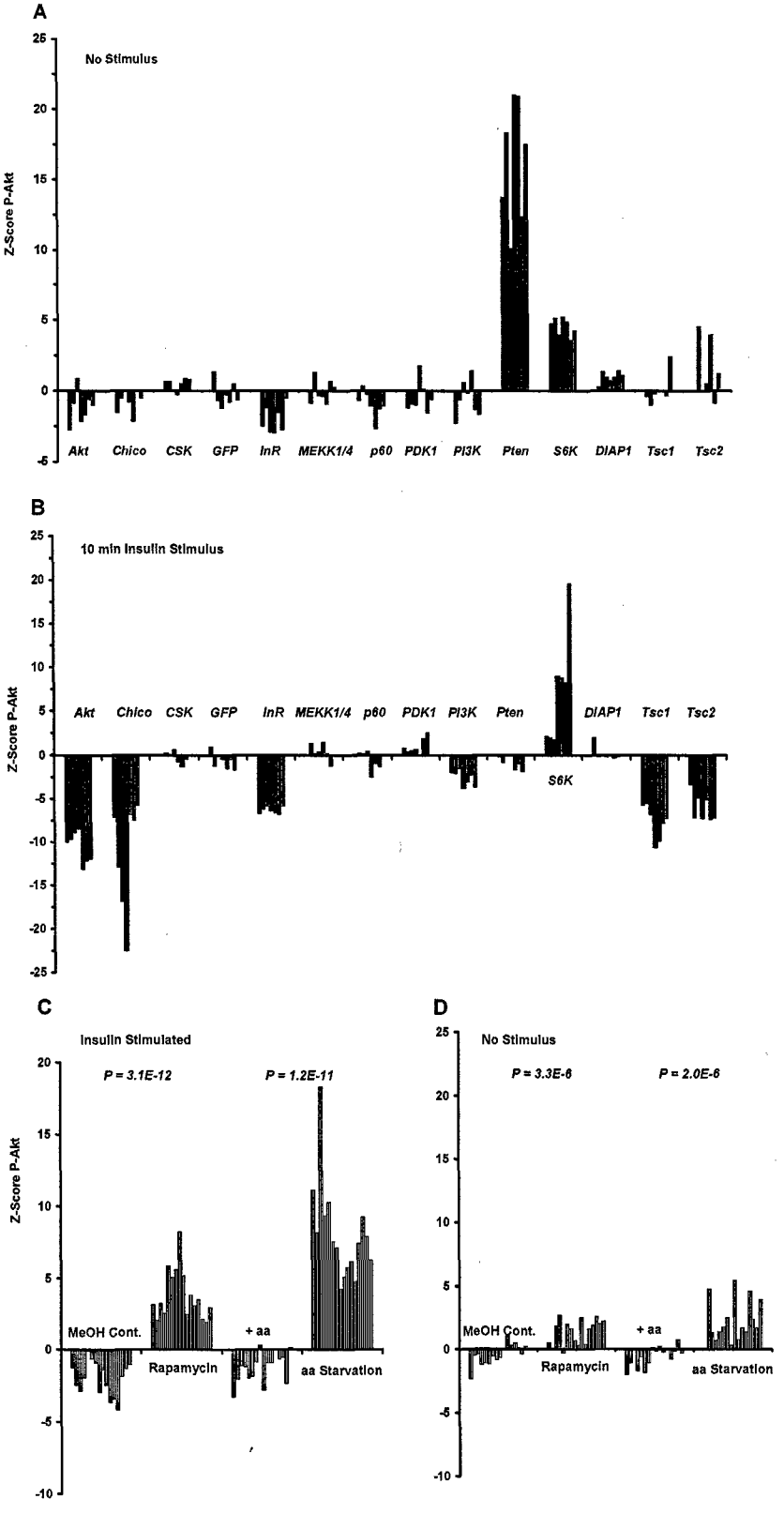

A
No Stimulus
Z-Score P-Akt
Akt Chico CSK GFP InR MEKK1/4 p60 PDK1 PI3K Pten S6K DIAP1 Tsc1 Tsc2
B
10 min Insulin Stimulus
Z-Score P-Akt
Akt Chico CSK GFP InR MEKK1/4 p60 PDK1 PI3K Pten S6K DIAP1 Tsc1 Tsc2
C
Insulin Stimulated
P = 3.1E-12
P = 1.2E-11
Z-Score P-Akt
MeOH Cont.
Rapamycin
+ aa
aa Starvation
D
No Stimulus
P = 3.3E-6
P = 2.0E-6
MeOH Cont.
Rapamycin
+ aa
aa Starvation

Figure 4

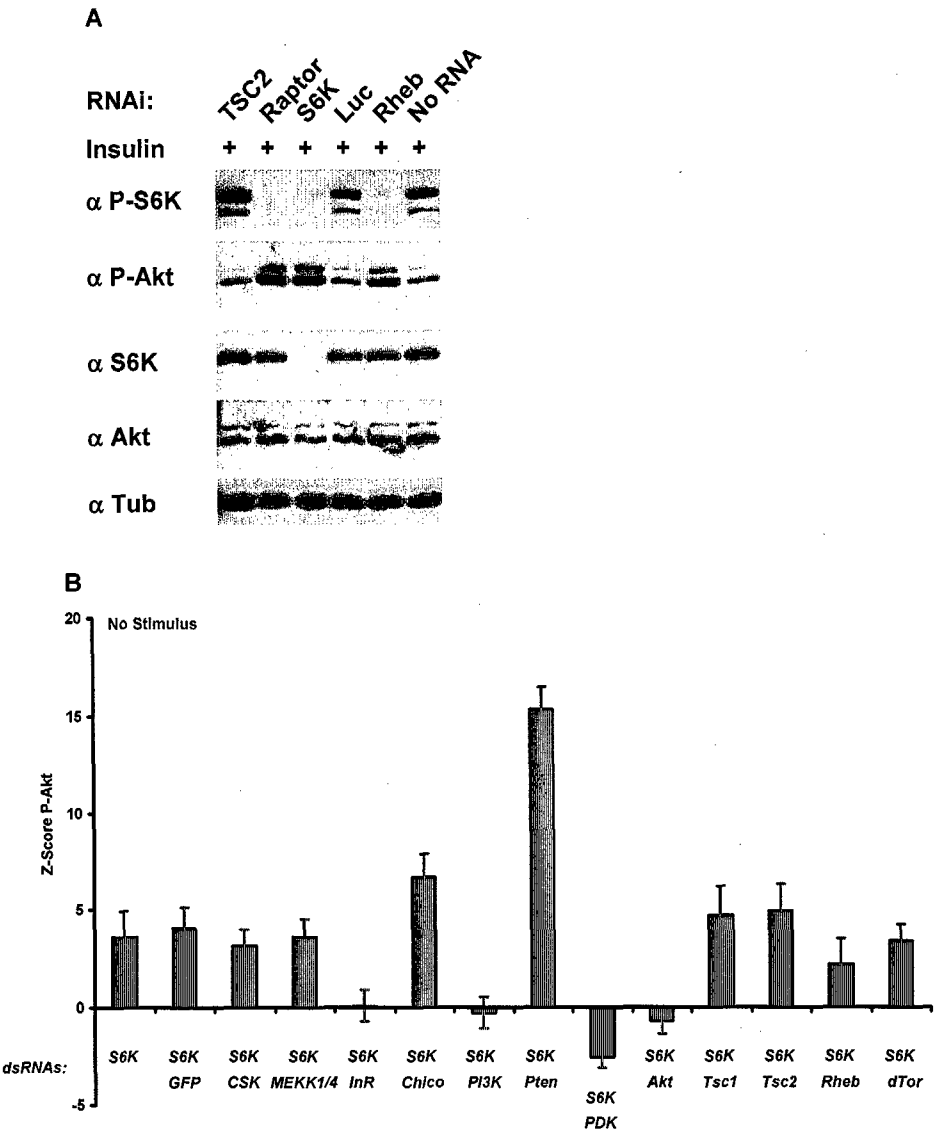
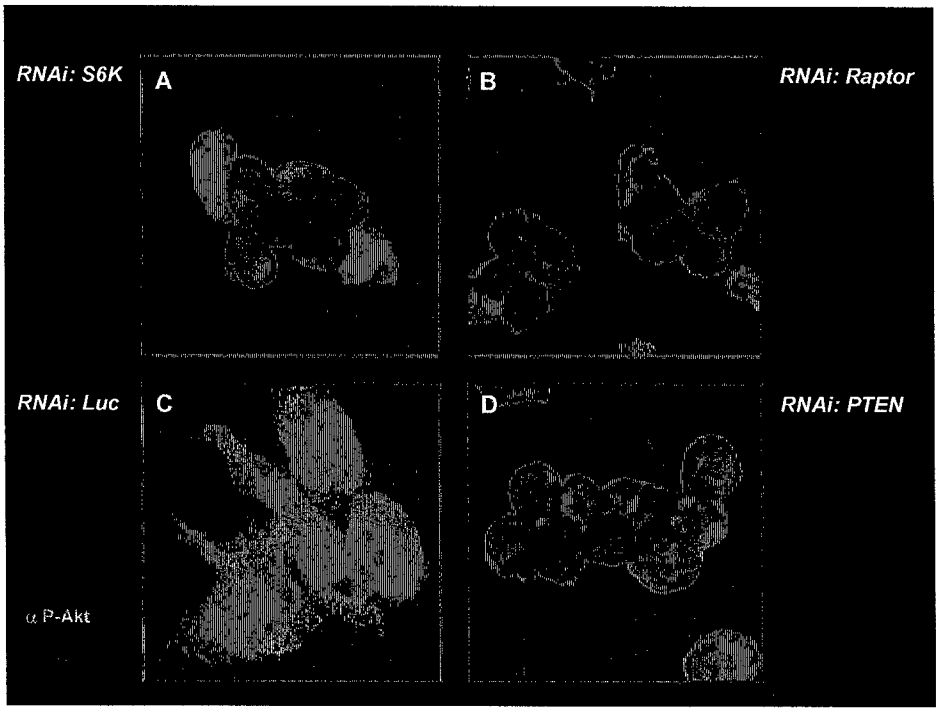

A
RNAi:
TSC2
Raptor
S6K
Luc
Rheb
No RNA
Insulin
+ + + + + +
α P-S6K
α P-Akt
α S6K
α Akt
α Tub
B
No Stimulus
Z-Score P-Akt
20
15
10
5
0
-5
dsRNAs:
S6K
S6K GFP
S6K CSK
S6K MEKK1/4
S6K InR
S6K Chico
S6K PI3K
S6K Pten
S6K PDK
S6K Akt
S6K Tsc1
S6K Tsc2
S6K Rheb
S6K dTor

Figure 5


RNAi: S6K
A
B
RNAi: Raptor
RNAi: Luc
C
D
RNAi: PTEN
α P-Akt

E

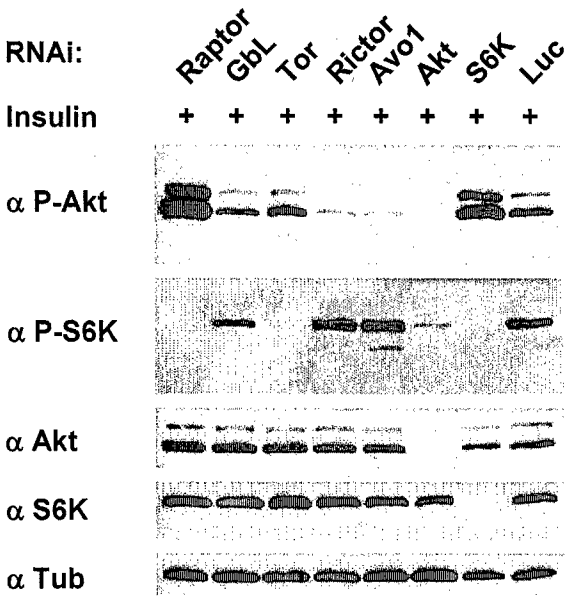

RNAi:
Raptor
GbL
Tor
Rictor
Avo1
Akt
S6K
Luc
Insulin
+ + + + + + + +
α P-Akt
α P-S6K
α Akt
α S6K
α Tub

Figure 6

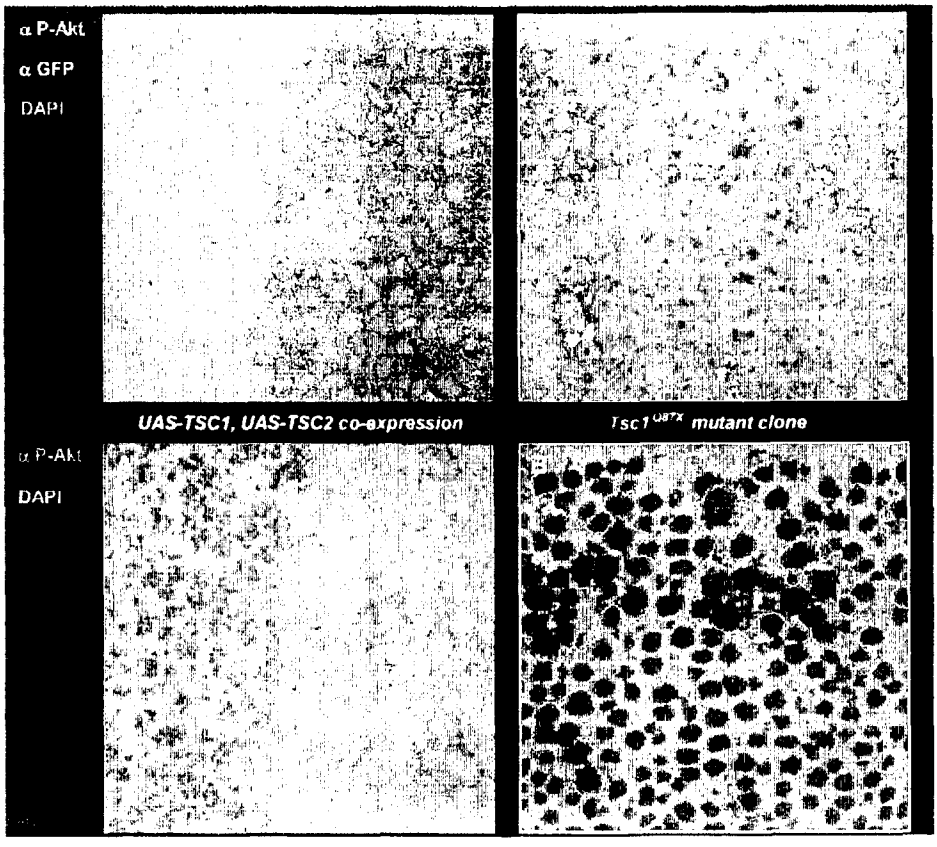

α P-Akt
α GFP
DAPI
UAS-TSC1, UAS-TSC2 co-expression
Tsc1 QA77 mutant clone
α P-Akt
DAPI

Figure 7

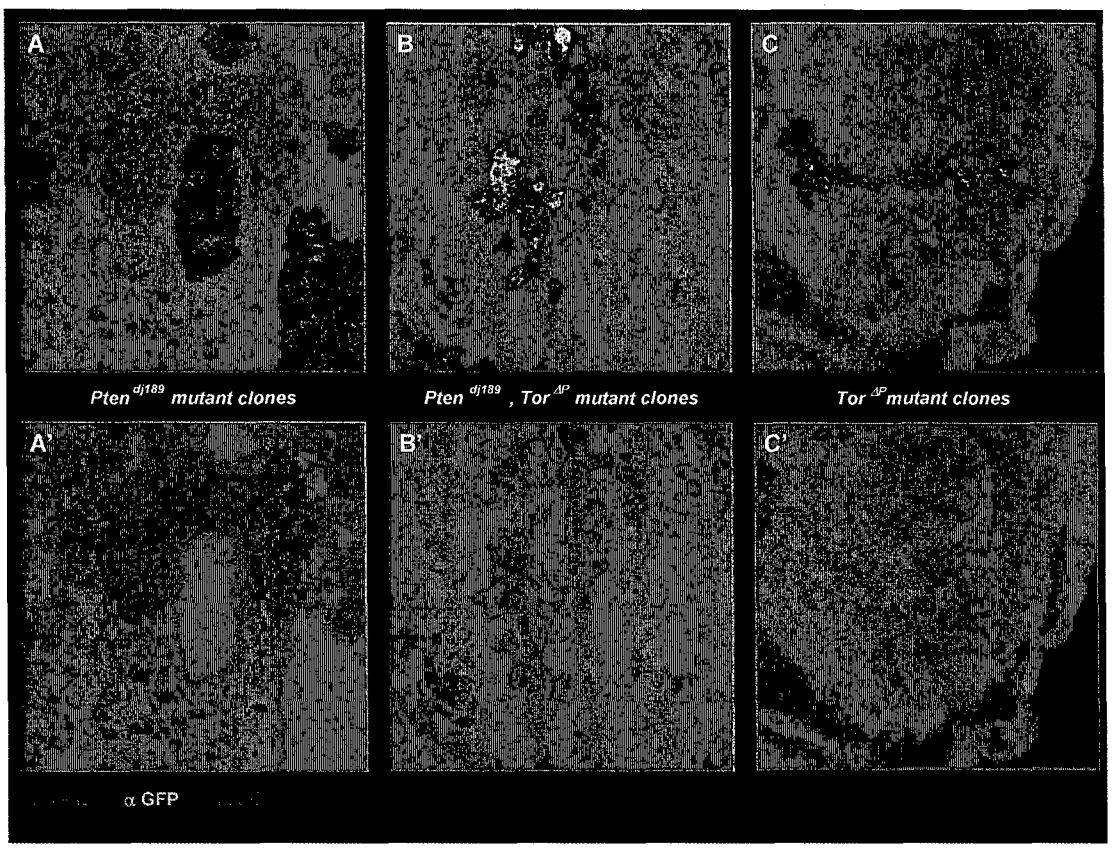
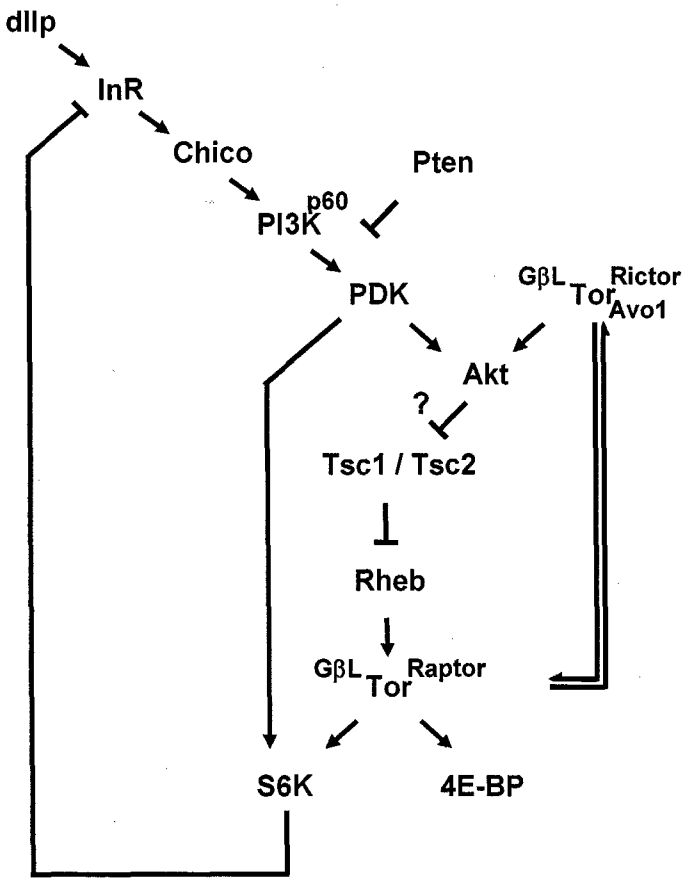

A
B
C
Pten dj189 mutant clones
Pten dj189 , Tor ΔP mutant clones
Tor ΔP mutant clones
A'
B'
C'
α GFP

Figure 8


dIlp
InR
Chico
Pten
PI3K p60
PDK
GβL Tor Rictor Avo1
Akt
?
Tsc1 / Tsc2
Rheb
GβL Tor Raptor
S6K
4E-BP